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DOES THE BULL SPREAD INFECTIOUS ABORTION IN CATTLE?

EXPERIMENTAL STUDIES FROM 1936 TO 1942*

By

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INTRODUCTION

Two previous reports of experimental studies have been published by the writer throwing some light on copulative transmission of infectious abortion among cattle. For many years this problem has occupied the attention of veterinary pathologists especially in the United States of America, but the question has not been settled conclusively.

From the work published in the first of the above reports (Thomsen, 1928) taken in conjunction with the experimental results recorded by other investigators, it was evident that the original view could no longer be maintained that infectious abortion is a disease chiefly spread by the bull during copulation. The experiments cited, and in particular those reported from U.S.A., where attempts at copulative transmission were made directly upon cows which afterwards became fertilised, had given negative results; the same was found to apply in corresponding experiments carried out by the writer on goats. These results suggested that, in practice, the role of the male in the direct transmission of infectious abortion was insignificant; the spread of infection through copulation could, therefore, be disregarded.

In the second paper (Thomsen, 1936), preliminary experiments with infected bulls were described. These were planned keeping in mind that it was of prime importance to discover whether a bull that had served an aborted cow shortly after the act of abortion was apt to convey the infection on subsequent service of healthy cows. The bull was, therefore, massively infected immediately before serving the experimental heifers. A thick suspension of infected placental exudate (in physiological salt solution) was introduced into the preputial sac, so that the bull was bound to convey a large number of *Br. abortus* to any heifer served immediately after such treatment. A total of 15 heifers was served in this manner. As a result, five heifers aborted, five calved normally and five failed to become pregnant. Four control heifers, placed with the above, calved normally. In spite of the massive transmission, one-half of the heifers which became pregnant calved normally. In natural circumstances such massive infection is unlikely to occur as the aborting

*Lecture delivered at the Royal Veterinary High School, Stockholm, February 13th, 1943.

cow in a few days contains few microscopically demonstrable *Br. abortus* in her vaginal discharge and usually gets rid of the bacilli completely within a period which may extend from a few weeks to two months. As a rule the bull will, therefore, be exposed only to comparatively slight infection when it serves a cow which has aborted. It thus seemed desirable to prosecute further experiments planned to resemble more closely normal conditions.

A. EXPERIMENTS TO ASCERTAIN WHETHER NATURALLY INFECTED
BULLS CAN TRANSMIT INFECTION DURING COPULATION

Bulls with Unilateral Brucella orchitis

Although the practising veterinary surgeon is not likely to meet it often in his practice, *Brucella* infection of the bull's testes, with intense swelling and tenderness, is not a rare disease and is usually unilateral. As to the frequency of the condition in Denmark, a fair impression was obtained from an enquiry addressed in April, 1938, to all veterinary surgeons in the country outside the City of Copenhagen. A questionnaire was circulated asking those acquainted with suspected cases of *Brucella orchitis* to give the laboratory the opportunity to buy these bulls. Offers of eight such bulls were received. Although it is improbable that all the animals known to have *Brucella orchitis* were offered for sale, it is reasonable to assume that there were not many more such cases in the country at that time, for it may be taken that veterinary surgeons would readily co-operate with the laboratory in obtaining such material, particularly as they were able to offer their clients economically advantageous terms. Even if this lesion is not of frequent occurrence, the affected animal—if it does, in fact, transmit infection and especially if it is a "co-operative bull"—may give rise to extensive damage.

EXPERIMENT I

This experiment was carried out with a bull of the Jutland breed, two years old, weighing about 700 kg., obtained from a herd in Jutland in which abortion was occurring. It was received at the laboratory on July 22nd, 1936. About three weeks previously, the owner of the bull had noticed that its right testis was swollen and the swelling increased markedly during the following days. The onset of the condition was associated with loss of appetite, which lasted for some days, and there was also evidence of pain in the enlarged testicle. During the following days the bull served several cows, but not very willingly. On July 11th, the agglutination titre of the bull's serum was 1:200, and the complement fixation titre 1:20. On arrival at the laboratory, the right testis was greatly swollen, but there was no tenderness or increased heat. A specimen of the semen collected in an artificial vagina on July 24th, revealed no *Br. abortus* on microscopic examination, in cultures, or on inocula-

tion of two g. pigs. At this time the bull served readily, but the semen was less opaque than normal, with relatively scanty spermatozoa. A further specimen of semen was obtained on August 4th, and the presence of *Br. abortus* was demonstrated in it by inoculation of g. pigs. On August 18th, semen was again examined and a few organisms were demonstrated in cultures. The specimen was of more normal appearance, though still containing some mucous floccules. Colony counts corresponded to 100 bacteria per c.c. of semen.

The bull was now employed for the service of 15 reaction free, sexually mature heifers purchased from abortion-free herds, either by copulation or by artificial insemination of semen. The data on the individual heifers are presented in the following protocols:—

Heifer No. 333.—12.9.36: Bull's semen collected in artificial vagina (two jumps*), yield 9 c.c. Cultures showed growth of *Br. abortus* indicating about 2,000 per c.c. of semen. One of two g. pigs killed one month after inoculation was positive. The semen was thin. Artificial insemination of heifer with 5.5 c.c. of semen (in three portions). 12.1.37: rectal examination showed no pregnancy. Heifer sold. Monthly blood examinations, no reaction.

Heifer No. 334.—19.9.36: Semen collected (two jumps), yield 6 c.c. No *Br. abortus* demonstrable on cultivation or g. pig inoculation, but this does not necessarily exclude the presence of *Br. abortus*—see Heifer No. 339. *Br. abortus* demonstrated in the semen on 12.9 and 23.9. Artificial insemination with 3 c.c. of semen. 12.1.37: rectal examination showed no pregnancy: heifer sold. Monthly blood examinations, no reaction.

Heifer No. 335.—19.9.36: Semen collected (two jumps), yield 6 c.c. No *Br. abortus* demonstrable on cultivation or g. pig inoculation. Served normally by the bull and 1 c.c. of semen injected into the cervical canal. 12.11.36: oestrus recurred. Heifer sold. Monthly blood examinations, no reaction.

Heifer No. 339.—23.9.36: Semen (two jumps), yield 3.5 c.c. and 2.5 c.c. No growth of *Br. abortus* in cultures from the two ejaculates, but of the two g. pigs inoculated with the same ejaculates, one developed brucellosis. (N.B.—This shows that semen may contain *Br. abortus* even when no growth is obtained in cultures and when the g. pigs inoculated from a single ejaculate, are not infected. These animals were killed after one month.) Heifer served normally. 18.6.37: normal calving; placenta not infected. Monthly blood examinations from September, 1936, to June, 1937, gave completely negative agglutination and complement fixation reactions.

Heifer No. 340.—25.9.36: Semen lost first jump, third jump 2 c.c. of rather thin semen collected, containing many spermatozoa. *Br. abortus* isolated by cultivation. No infection in two g. pigs (killed after one month). Heifer served normally (second jump). 12.1.37: rectal examination showed no pregnancy. Heifer sold. Monthly blood examinations, no reaction during experimental period.

Heifer No. 310.—29.9.36: Semen collected first jump, yield 2 c.c.; third jump, similar to the first. No *Br. abortus* demonstrable by cultivation

* As a rule, the interval between the jumps mentioned in the protocols has been about ten minutes.

and inoculation (two g. pigs killed after one month): *Br. abortus* demonstrated in the semen on 25.9 and 3.10. Heifer served normally on 29.9.36 (second jump). 22.12.36: oestrus. 12.1.37: rectal examination, showed no pregnancy. Heifer sold. Monthly blood examinations, no reaction throughout.

Heifer No. 333.—30.9.36: Semen collected first jump, yield 1.5 c.c.; third jump similar to first. No *Br. abortus* demonstrable by cultivation and inoculation (two g. pigs), but bacilli were demonstrated in the semen on 25.9 and 3.10. Heifer served by bull on 30.9.36 (second jump). 10.7.37: normal calving; placenta not infected. Monthly blood examinations, from September, 1936 to July, 1937, were completely negative.

Heifer No. 337.—3.10.36: Semen collected first jump, yield 1.5 c.c. of thin semen; third jump similar to first. Cultures from both specimens gave growth of *Br. abortus*. No infection in two g. pigs. Heifer served normally (second jump) 12.1.37: rectal examination showed no pregnancy. Heifer sold. Monthly blood examinations: no reaction throughout.

Heifer No. 336.—26.11.36: Semen collected first jump, yield 1 c.c. of thin semen; third jump, 3 c.c. of normal-looking semen collected. *Br. abortus* demonstrated in both ejaculates by g. pig inoculation (infection in two out of three g. pigs). Heifer served normally (second jump). 1.9.37: normal calving; placenta not infected. Monthly blood examinations, from November, 1936, to September, 1937, completely negative.

Heifer No. R.25.—28.1.37: Semen collected at second jump (three hours after first). *Br. abortus* not demonstrable on cultivation or g. pig inoculation. Heifer served normally (first jump). Service repeated three hours later (third jump). 20.5.37: not pregnant. 21.5.37: semen collected. *Br. abortus* demonstrated by cultivation. Placed in fold* together with the bull and four other heifers till 3.9.37 (then pregnant). 23.5.37: estimated day of fertilisation. 27.2.38: normal calving (twins); placenta not infected. Monthly blood examinations from January, 1937, to February, 1938, negative throughout.

Heifer No. R.26.—20.2.37: Semen collected at second jump (three hours after first). *Br. abortus* not demonstrable in this specimen on cultivation or inoculation, but demonstrated on 2.3.37. Heifer served normally (first jump); service repeated three hours later (third jump). 20.5.37: pregnant. 5.12.37: normal calving; placenta eaten by the cow. Monthly blood examinations from January, 1937, to January, 1938, completely negative throughout.

Heifer No. R.27.—2.3.37: Semen collected at second jump (three hours after first). *Br. abortus* demonstrated by g. pig inoculation 2.3.37. Heifer served normally (first jump); service repeated three hours later (third jump). 20.5.37: not pregnant. Placed in fold with the bull and four other heifers until 3.9.37, then pregnant. 11.6.37: estimated day of fertilisation. 18.3.38: normal calving; placenta not infected. Monthly blood examinations from January, 1937, to March, 1938, negative throughout.

Heifer No. R.28.—11.3.37: Semen collected at second jump (two and a half hours after first). Numerous *Br. abortus* (about 1,000 per c.c. of semen) demonstrated by cultivation, and on g. pig inoculation. Normal service (first jump); service repeated about two hours later (third jump). 19.5.37:

* This natural method was adopted because "service on schedule" was found to give too low a percentage of fertilisation.

in oestrus. 20.5.37: rectal examination, showed no pregnancy. 21.5.37: semen collected. *Br. abortus* demonstrated by cultivation. Placed in fold with bull and four other heifers until 3.9.37 (then pregnant). 21.5.37, estimated date of fertilisation. 25.2.38: normal calving; placenta not infected. Monthly blood examinations from January, 1937, to February, 1938, negative throughout.

Heifer No. R.29.—31.3.37: Semen collected at second jump (45 mins. after first), yield 5 c.c. *Br. abortus* demonstrated by cultivation (about 500 per c.c. of semen) and by g. pig inoculation. Heifer served normally (first jump); service repeated one hour later (third jump). 7.5.37: in oestrus. 20.5.37: rectal examination, showed no pregnancy. 21.5.37: semen collected, gave growth of *Br. abortus* in cultures. Placed in the fold with bull and four other heifers, until 3.9.37, then pregnant. 21.6.37: estimated day of fertilisation. 28.3.38: normal calving; placenta not infected. Monthly blood examinations from January, 1937, to March, 1938, negative throughout.

Heifer No. 443.—21.5.37: Semen collected; growth of *Br. abortus* in cultures. 22.5.37: placed in fold with bull and four other heifers until 3.9.37 (then pregnant). 14.6.37: estimated date of fertilisation. 21.3.38: normal calving; placenta not infected. Monthly blood examinations from May, 1937, to March, 1938, negative throughout.

From the above protocols it will be seen that nine of the 15 heifers served by a bull suffering from *Brucella orchitis* calved normally, while the remaining six failed to conceive. There was no instance of abortion among the 15 heifers and no blood reaction indicative of *Brucella* infection at any time. Nevertheless, it cannot be questioned from the data presented that the bull was excreting *Br. abortus* in its semen throughout the experimental period. Furthermore, it should be mentioned that the semen of this bull was found by cultural methods to contain *Br. abortus* as late as 9.3.38. At autopsy (26.4.38), a culture was obtained from the right epididymis, while the corresponding testis was the site of typical *Brucella* lesions. The right seminal vesicle was considerably larger than the left, but no growth was obtained in cultures from this organ. Again the serum of the bull gave a strong *Brucella* reaction throughout the experimental period. The last test, on 28.1.38, showed an agglutination titre of 1:200, and a complement fixation titre of 1:20. On those occasions when *Br. abortus* bacilli could not be demonstrated, the semen, despite its collection in a sterilised artificial vagina, was soiled by the penis and often contained so many contaminating bacteria that the culture plates were overgrown by them. Moreover, as is well known, infection is obtained in g. pigs only when relatively large numbers of bacteria are inoculated. On the other hand, it is unsafe to employ larger quantities of semen for inoculation, as g. pigs do not withstand this treatment well. In these experiments the invariable practice was to inject 0.5 c.c. of semen subcutaneously.

Two control heifers, served by a non-infected bull, stabled side by side with two of the experimental animals, failed to show any sign of infection. This result is not surprising.

EXPERIMENT II

In order to supplement the above findings, experimental work was continued with more bulls during 1938-39. Eight additional bulls with presumed unilateral *Brucella* orchitis were obtained: of these only three were found suitable for experiment, as one was suffering from tuberculous orchitis, three showed aspermia, and one did not excrete *Br. abortus* in the semen. Concerning the three bulls employed, the following data are available:—

Bull 448 was of the Jutland breed, two and a quarter years old, weighing 550 kg. An orchitis of the right testicle was observed on April 4th, 1938, with marked local inflammatory symptoms. Body temperature 40.1°C . Appetite poor. Blood examination on April 30th showed an agglutination titre of 1:200; and a complement fixation titre of 1:200. On 1.6.38, semen was collected by the artificial vagina method, 3 c.c. of a thin floccular, rapidly sedimenting semen being obtained. Microscopic examination (hanging drop and stained smear) showed the spermatozoa count to be somewhat below normal, but otherwise there was no abnormality. *Br. abortus* was demonstrated by cultivation. Specimens of semen were examined until 13.8.38, and *Br. abortus* was demonstrated by cultivation on 10.6, 9.7 and 11.7.38.

Bull 449 was a Shorthorn, two and a half years old, weighing about 650 kg. An orchitis of the right testicle, with marked local inflammatory symptoms, was first observed April 25th, 1938. Blood examination on April 29th showed an agglutination titre of 1:1,000; complement fixation titre 1:1,000. On 12.5.38, semen was collected and 4 c.c. of thin fluid obtained. Microscopic examination revealed no abnormality. *Br. abortus* was not demonstrable in this specimen. Subsequently, samples of semen were examined from time to time, and *Br. abortus* was demonstrated on 16.6 and 11.7.38.

Bull 451 was of the Red Danish breed, three years old, weighing 682 kg. A clinical examination on June 16th, 1938, revealed a swelling of the left testicle, which was said to have been present from three to four months, without any other signs of illness being noticed. Except for the swelling no signs of inflammation were observed, and the bull served willingly. A blood sample showed an agglutination titre of 1:200; complement fixation titre, 1:10. On 24.6.38, semen was collected, yielding 5 c.c. of rather watery fluid. Microscopic examination showed no abnormality, and *Br. abortus* was not demonstrated. Until the middle of August, 1938, several semen samples were examined, but *Br. abortus* was found on June 29th only.

All three bulls served willingly.

To give infection by copulation the greatest possible chance of manifesting itself, these *Brucella*-excreting bulls were given ample opportunity to serve a group of twelve reaction-free heifers purchased from infection-free herds. Commencing on August 13th, 1938, the heifers were placed in a fenced pasture* together with the bulls, in rotation, one at a time. The bulls which were each hitched to a drag-tether, were changed three times in the day. It was reasonable

* In order to obtain a satisfactory conception rate it was found necessary to allow the animals to copulate freely although this made the control of the bacillary content of the semen less effective.

to expect that each heifer when in oestrus would be served by each bull, possibly several times, and thus be exposed to rather heavy infection. The attendant believed that each heifer was served in this way about ten times in each oestral period.

On rectal examination by Professor Folmer Nielsen†, on September 21st, 1938, the heifers Nos. 453, 454, (?), 456, (?), 457, 463, 464 and 465 were found probably to be pregnant. The heifers were then stabled, two in a stall, so that none was completely isolated. On October 27th, Nos. 455, 460, 461 (?) and 462 were found to be pregnant, whereas No. 458 was not in calf, and was excluded from the experiment. The pregnant heifers were stabled in the same manner as the preceding‡. The following table gives the details recorded subsequently upon the twelve heifers. (Table I.)

As shown in Table I, ten of the twelve heifers calved normally without demonstrable *Br. abortus* in the placenta. Two failed to conceive. In four of the animals a trace of antibody formation was found, indicating a slight transitory infection.

In this experimental group, it was thus impossible to obtain any evidence of infection transmitted by copulation resulting in abortion.

B. EXPERIMENTS WITH BULLS SERVING EXPERIMENTAL HEIFERS IMMEDIATELY AFTER COPULATION WITH RECENTLY ABORTED COW WHICH WAS EXCRETING *Br. abortus* IN THE UTERINE DISCHARGE

The experiments in this group were distributed over a considerable period, namely, 1939 to 1942. They were difficult and costly to carry out, because it is not easy at every stage to have available a suitable recent case of abortion as the source of infection, and also because it was considered more appropriate to allow copulation to conform to a schedule, a procedure which gives a lower percentage of conceptions.

In these experiments, three bulls were employed, five aborted cows, and 22 sexually mature experimental heifers. Two of these heifers were used as controls. They were served by a non-infected bull but stabled side by side with the other heifers. The bulls and heifers were purchased from healthy, reaction-free herds.

To reproduce so far as possible the type of copulative infection risk considered to be the most common, namely, infection transmitted by a bull which shortly beforehand has served a recently aborted cow, efforts were made to have available a "new aborter," the placenta of which had been shown to contain large numbers of

† The Serum Laboratory wishes to express its indebtedness to Professor Folmer Nielsen for his frequent and ever-ready assistance in the examination of the heifers for pregnancy.

‡ The bulls were slaughtered at this point. In all three, *post-mortem* examination revealed changes characteristic of *Brucella orchitis*, while the accessory glands appeared macroscopically to be normal. Neither cultures from the sexual glands (including the accessory glands) nor inoculation of g. pigs with material from them revealed *Br. abortus*. At the time of slaughter the serum of bulls 448 and 449 gave an agglutination titre of 1 : 500, and that of bull 451, 1 : 200.

TABLE I

THE GESTATIONAL HISTORY AND SEROLOGICAL DATA ON THE 12 HEIFERS
IN EXPERIMENT II (1938-1939)

<i>Heifer No.</i>	<i>Running Period</i>	<i>Pregnancy and Calving</i>	<i>Monthly Serological Tests</i>
453	13.8-21.9	Calved normally on 30.5. Placenta not infected, delivered spontaneously (examined macroscopically, microscopically, and by Holth's antigen test)	Aug. to Oct., Jan., Feb. : Ag. 0* ; Cp. 0† Mar. : Ag. 1 : 20, partial ; Cp. 0 Apr. : Ag. 0 ; Cp. 0 May : Ag. 1 : 20, partial ; Cp. 0 June : Ag. 0 ; Cp. 0
454	13.8-21.9	Oestrus recurred on 5.1.39. Rectal examination, not pregnant. (Possibility of non-observed abortion very improbable.) Excluded from experiment	Aug. to Dec. : Ag. 0 ; Cp. 0 Jan. 24 : Ag. 0 ; Cp. 1 : 10, partial
455	13.8-27.10	Calved normally on 21.6. Placenta not infected, delivered spontaneously	Aug. to Dec., Jan. to Aug. : Ag. 0 ; Cp. 0
456	13.8-21.9	Calved normally on 19.5. Placenta not infected, but retained	Aug. to Dec., Jan. to June : Ag. 0 ; Cp. 0
457	13.8-21.9	Calved normally on 22.5. Placenta not infected	Aug. to Dec., Jan. to June : Ag. 0 ; Cp. 0
458	13.8-27.10	Rectal examination on 27.10, not pregnant. Excluded from the experiment	Aug. to Oct., Ag. 0 ; Cp. 0
460	13.8-27.10	Calved normally on 16.6. Uterus not infected, placenta eaten by the animal. Animal slaughtered	Aug. Ag. 0 ; Cp. 0 Sept., Oct., Dec., Ag. 0 ; Cp. 1-10, partial Jan. to June 16 : Ag. 0 ; Cp. 0
461	13.8-27.10	Calved normally on 13.7. Placenta not infected	Aug. to Dec., Jan. to July 13 : Ag. 0 ; Cp. 0
462	13.8-27.10	Calved normally on 7.6	Aug. to Dec., Jan. to June 7 : Ag. 0 ; Cp. 0
463	13.8-21.9	Calved normally on 24.5. Placenta not infected	Aug. to Dec., Jan. to June : Ag. 0 ; Cp. 0
464	13.8-21.9	Calved normally on 21.5. Placenta not infected	July : Ag. 0 ; Cp. 0 Aug. Sept. : Ag. 0 ; Cp. 1 : 20 Oct., Dec., Jan. : Ag. 0 ; Cp. 0 Feb. : Ag. 0 ; Cp. 1 : 20 Mar. to June : Ag. 0 ; Cp. 0
465	13.8-21.9	Calved normally on 31.5. Placenta not infected	Aug. to Dec., Jan. to June : Ag. 0 ; Cp. 0

*Ag = Agglutination titre. †Cp = Complement fixation titre.

Br. abortus. As a rule, these were abortion cases purchased from newly infected herds. When one of the experimental heifers showed oestrus, it was allowed service by a bull which had covered the aborting cow immediately beforehand. Before the service of this aborting cow, or immediately after, discharge was obtained from the vagina, using a vaginal spoon, for bacteriological examination.

Details concerning this experimental series are given in the following Table (Table II). It should be pointed out here, however, that eight of the heifers failed to conceive, and for this reason were excluded from the experiment.

From Table II it is evident, among other things, that none of the heifers exposed to infection by copulation with normal bulls that had just served a cow that had aborted, acquired an infection which resulted in abortion. Nor did any of these pregnant heifers give a positive serological *Brucella* reaction at any time in the experimental period. On the other hand, two of the eight non-pregnant heifers (Nos. 402 and 419) showed a transitory trace of reaction indicating a slight infection which failed to become established. Table II furthermore shows that all the pregnant heifers calved normally, including the two controls, and also that two of the experimental heifers came into oestrus during pregnancy and were thus served by contaminated bulls while pregnant. Of the bulls employed for transmission of the infection, one remained reaction-free throughout the service period, November, 1939 to August, 1940, whereas the other used on infected cows during the period September to November, 1940, gave a weak reaction on November 4th (Ag. 1 : 20 ; Cp. 0).

DISCUSSION

In the more recent veterinary literature the subject under investigation appears to have been only cursorily dealt with so far as the experimental aspects of the problem are concerned. In Denmark, Jepsen and Jorgensen (1938) have dealt with spontaneous *Brucella* orchitis and chronic inflammation of the seminal vesicles in seven bulls. The bulls served altogether 14 normal heifers, ten of which became pregnant. All ten heifers calved normally, and none showed infection of the placenta. Serological tests showed repeatedly a negative reaction throughout the experimental period. The outcome of their experiments is quite in harmony with the results here presented, as these authors also were unable to demonstrate that infection was transmitted during copulation by the seven bulls employed. They believe, however, that bulls with acute *Brucella* inflammation of the seminal vesicles may behave differently. Another interesting, although somewhat older, work should be mentioned in this connection, namely that of Lubbehusen and Fitch (1926). These authors give a valuable survey of the problem besides presenting the view generally held upon the question in U.S.A. and state: "Granting that a small percentage of reacting bulls do discharge *Bacterium abortus* Bang in the semen, there is much experimental evidence to

TABLE II
DETAILS CONCERNING THE GESTATIONAL HISTORY AND SEROLOGICAL EXAMINATIONS OF THE HEIFERS IN EXPERIMENT III. 1939/1942.

Heifer No.	Copulation	Bacteriological Examination of Vaginal Discharge, etc., from Aborting Cow	Calving	Monthly Serological Tests	Remarks on Abortion Case Used
408	26.12.39, immediately after the bull had served <i>Abortion Case I</i> (pro-fuse vaginal discharge)	I. <i>Br. abortus</i> in vaginal discharge on 3.1 and 6.1, and in uterine contents on 9.1.40	Calved normally on 9.10.40. Placenta not infected (macroscopic and microscopic examination and Holth's antigen test)	Dec. '39, Jan. to Nov.; Ag. 0*; Cp. 0†	<i>Abortion Case I</i> had aborted on 19.12.39. First abortion in the herd followed by many more
407	3.1.40, immediately after the bull had served <i>Abortion Case I</i> (vaginal discharge)	See 408	Calved normally on 16.10.40. Placenta not infected.	Dec. '39, Jan. to Oct. 26; Ag. 0; Cp. 0	
404	6.1.40, immediately after the bull had served <i>Abortion Case I</i> (vaginal discharge). 19.2.40. In oestrus, served again after the bull had covered <i>Abortion Case III</i> (vaginal discharge)	See 408 III. <i>Br. abortus</i> in vaginal discharge on 10.2.40	Calved normally on 12.10.40 (pregnant to service on 6.1). No infection in uterus or placenta	Dec. '39, Jan. to Sept.; Ag. 0; Cp. 0	<i>Abortion Case III</i> had aborted on 20.1.40; abortion prevailing in the herd
406	15.1.40, immediately after the bull had served <i>Abortion Case II</i> (pro-fuse vaginal discharge)	II. <i>Br. abortus</i> in vaginal discharge on 15.1.40	Calved normally on 26.10.40. Placenta not infected	Jan. to Nov.; Ag. 0; Cp. 0	<i>Abortion Case II</i> had aborted on 5.1.40; first abortion in a herd of 4 cows
409	10.2.40, in oestrus, served immediately after the bull had covered <i>Abortion Case III</i>	See 404	Calved normally on 22.4.40. (The heifer was therefore pregnant when served on 10.2.40.) Placenta not infected	Jan. to May 25; Ag. 0; Cp. 0	
427	20.9.41, immediately after the bull had served <i>Abortion Case A</i> (pro-fuse vaginal discharge). 16.10.41, and 4.11.41 again in oestrus. Served immediately again after the bull had served <i>Abortion Case B</i>	<i>Br. abortus</i> in vaginal discharge on several occasions between 19.9 and 22.11.41 and in uterine scrapings on 12.10 and 11.12.41	Calved normally on 19.7.42. Placenta not infected. (Pregnant after copulation on 16.10.41)	Sept. '41 to Dec., Feb. to July 20; Ag. 0; Cp. 0	<i>Abortion Case A</i> had aborted on 8.9.41. First abortion in herd; no abortion since (9 animals) <i>Abortion Case B</i> had aborted on 15.10.41. First abortion in the herd; since then abortion prevailing

428	20.9.41, immediately after the bull had served <i>Abortion Case A</i> (profuse vaginal discharge). 11.10.41 and 22.11.41 in oestrus again. Served just after the bull had served <i>Abortion Case B</i>	See above	Calved normally on 27.8.42. Placenta not infected	Sept. '41 to Dec., Feb. to Aug. 29; Ag. 0; Cp. 0
423	23.9.41, immediately after the bull had served <i>Abortion Case A</i>	<i>Br. abortus</i> in vaginal discharge on 23.9 and several other times	Calved normally on 2.7.42. Placenta not infected	Sept. to Dec., Feb. to July 16; Ag. 0; Cp. 0
426	2.10.41, immediately after the bull had served <i>Abortion Case A</i>	<i>Br. abortus</i> in vaginal discharge on 2.10	Calved normally on 15.7.42. Placenta not infected	Sept. to Dec., Feb. to Aug. Ag. 0; Cp. 0
420	14.10.41 and 4.11.41, immediately after the bull had served <i>Abortion Case B</i> (profuse vaginal discharge)	See 427	Calved normally on 8.8.42. Placenta not infected	Sept. to Dec., Feb. to Aug. 11; Ag. 0; Cp. 0
422	17.10.41, shortly after the bull had served <i>Abortion Case B</i>	See 427	Calved normally on 17.7.42. Placenta not infected	Sept. to Dec., Feb. to Aug. Ag. 0; Cp. 0
410	No sign of oestrus since her arrival at the experimental farm		Calved normally on 4.5.40. The heifer was therefore pregnant before purchase. Placenta not infected	Feb. to Dec., Jan. 24, '41; Ag. 0; Cp. 0
CONTROLS				
401 (in stall with 402)	10.2.40 and 26.4.40, served by a non-infected bull		Calved normally on 24.1.41. Placenta not infected	Feb. to Dec., Jan. 24, '41; Ag. 0; Cp. 0
403 (in stall with 404)	10.2.40, 29.2.40 and 20.3.40, served by a non-infected bull		Calved normally on 24.12.40. Placenta not infected	Feb. to Dec., Jan., '41; Ag. 0; Cp. 0

* Ag. = Agglutination titre.

† Cp. = Complement fixation titre.

question their ability to infect the female at the time of service, inasmuch as the female is infected with difficulty by way of the vagina. True, infection of the female may occur as readily through food contamination with abortion-infected seminal discharges as by any other means. In instances of this kind it is not a breeding problem but one of improper management and its occurrence can be precluded in the well-managed herd."

From the experiments reported in this paper it is evident that an infection resulting in abortion could not be produced in any of the sexually mature heifers employed, although *Br. abortus* were introduced, often in considerable numbers, into their genital tracts during the act of copulation. This is probably due to the fact that heifers are but slightly susceptible to *Brucella* infection during the period when they are in oestrus. Their behaviour in this respect corresponds to that of goats, as had been demonstrated in the author's previous experimental studies (Thomsen, 1928).

According to the findings in this communication, the question whether the bull in natural circumstances is a source of spread is to be answered as follows: the possibility of such transmission in particular cases cannot be denied categorically as experimental infection by copulation is feasible when massive doses of infectious material are employed (Thomsen, 1936), but it is safe to conclude that it is not the mode of infection against which efforts need to be directed in the prevention of the disease. The probability of infection becoming transmitted through the bull in the sense ordinarily meant, that is by copulation, is only very slight. Yet in certain cases infection associated with service has to be considered. If, for instance, a cow is taken for service to a bull belonging to a herd in which infectious abortion prevails, it may contract an external contamination with the excretions of cows that have aborted, or even with those of the bull itself. In this event, the cow on its return to its own premises will transmit infection. But, in such a case the bull is at most only a passive spreader of infection. Conversely, if a cow that is discharging *Br. abortus* is taken to be served by the bull of a clean herd, infection may be transmitted passively.

SUMMARY

In the introduction a brief account is given of the experiments previously carried out on the experimental farm of the State Serum Laboratory, Copenhagen, in studies on the problem of the transmission of infectious abortion by the male during copulation.

Experiments now recorded fall into two main groups:—

1. In a study of transmission of infection, four bulls excreting *Br. abortus* in their semen were employed for the service of 27 sexually mature heifers. None of the heifers aborted; 19 calved normally, and none showed any evidence of placental infection; eight failed to conceive.

2. In experiments on the transmission of infection by bulls serving heifers immediately after copulation with a recently aborted cow excreting *Br. abortus* in the uterine discharge, 22 sexually mature heifers were used, the three bulls being contaminated by five aborted cows. None of the heifers aborted; 14 calved normally, and their placentae showed no evidence of infection; two of the 14 heifers were already pregnant at the time they were served by the infected bulls; eight heifers failed to conceive.

These experiments further invalidate the view that copulation is of significance in the spread of infectious abortion in cattle. Infection resulting in abortion could not be demonstrated in any instance.

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PARTURITION IN THE MARE

REPORT BASED ON OBSERVATIONS MADE IN NINE NORMAL CASES

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IN English veterinary literature records of detailed observations on the course of physiological parturition in the mare are scanty. The veterinary practitioner is seldom afforded the opportunity of witnessing the process from beginning to end, for unless he lives near the stud farm and can attend at a moment's notice, or is able and prepared to spend hours of waiting, it is probable the occasion will be missed, for once it becomes obvious to the attendant that labour has commenced, the birth of the foal is generally rapid. It is possible that the belief that the presence of observers disturbs the mare and hinders parturition may be responsible, in some measure, for this lack of detailed records. The cases under review did not support this belief.

This report is based on observations made by the writer in company with students of the Royal Veterinary College on eight Suffolk mares, involving nine parturitions, during the seasons 1940 and 1941.

The mares were frequently inspected during the later stages of gestation so that the progressive mammary development could be observed and the signs of approaching parturition studied. The stud-groom, who had great experience of foaling mares, and was particularly expert in detecting the signs of approaching labour, was instructed to notify the College the moment he considered parturition was at hand. In most cases the onset of patchy sweating was the sign looked for as evidence that uterine contractions had commenced.

Mammary Development.—The time before parturition when mammary hypertrophy became visible varied from 30 to 14 days. There seemed to be no significant difference between primiparae and multiparae in this respect. The influence of work on mammary enlargement was striking. The degree of swelling in a mare at grass, or one standing in, was much greater than in an animal at work. The swelling and tension which had become marked while a mare was standing in over the week-end was also in part lost when she was at work during the succeeding days. The mammary glands and teats afforded the best evidence of approaching parturition. About 48 hours before foaling it was noticed that the teats, when viewed from behind, were becoming altered in shape, appearing markedly swollen and tumefied, particularly at the base. By this time the width of each mammary gland, also viewed from behind, was $4\frac{1}{2}$ to 5 inches. Immediately before parturition it is usual for a little mammary secretion to escape from the teats and a pearly, wax-like bead to hang at the duct orifice. The onset of "waxing"

is accepted as good evidence of the nearness of parturition. Of the cases under observation one (multipara) did not show wax; this mare also did not sweat and her foaling took place at grass unobserved. In the remaining eight the earliest at which it was seen was 48 hours and the latest nine hours before parturition. When waxing occurred early, lactation commenced before the birth of the foal: as the mare moved, the pressure of her thighs against the enlarged glands caused milk to squirt out. The onset of lactation before parturition occurred in primiparae as well as multiparae.

TABLE I

Stud-book No.	Mare	Year of Birth	Parity	Hours before Foaling at which	
				(a) Wax first observed	(b) Lactation commenced
18147	Ridgeway Rosie ...	1935	Secundi :	9	—
18001	Marston Bloom ...	1934	Multi :	41½	20
15381	Tunstall Bounce (1940)	1929	"	48	24
19302	Ashmoor Mandolina ...	1936	Primi :	15½	—
18146	Ridgeway Rita ...	1935	Secundi :	30	—
15381	Tunstall Bounce (1941)	1929	Multi :	48	13
17417	Harkstead Ruby ...	1933	Secundi :	27	3
19912	Ridgeway Rambler ...	1937	Primi :	48	24
16821	Frimley Rebecca ...	1932	Multi :	—	—

The Vulva, Vagina, and Pelvic Ligaments.—The vulval labiae gave no indication of the nearness of parturition: neither, as in the cow, was there any tumefaction of the labiae, nor was parturition preceded by a flow of mucus; in fact the vaginal mucous membrane and the cervical canal remained "dry" and sticky until the onset of labour. (The mare "Bounce—1940" was examined *per vaginam* twelve hours before the onset of labour. The mucosa was sticky and tenacious. The cervix admitted three fingers and was occupied by sticky mucus which came away on the fingers.) No visible indication of approaching parturition was given by relaxation of the pelvic ligaments; it should be mentioned that the mares were in excellent condition and well rounded over the croup and buttocks.

Body Temperature and Pulse Rate.—On several occasions temperatures and pulse rates were taken during the last few days of gestation. The temperature showed no change from the normal and averaged 100·4° F. The pulse rate was accelerated and averaged 64 beats a minute. Similarly temperatures and pulses were taken during the first stage of labour. The temperature was found to be slightly subnormal, averaging 98·8° F., and the pulse rate averaged 64·5. (The writer understands that Benesch, F. (1941) has observed that the temperature of the cow becomes subnormal just before calving.)

Period of Gestation.—St. Cyr puts the average period of gestation in the mare as 346 days with normal variations of 307 to 394 days. Von Oettingen found the average duration of the Trakehnen breed to be 334 days and states that numerous foals of 315 to 320 days

became outstanding brood mares and sires. Williams, W. L. (1940) cites two Kentucky studs in which the average periods were 342.78 and 343.54 days.

In the cases under review the number of mares is insufficient to draw an average but it will be seen from Table II that there was a range of 317 to 363 days.

TABLE II

Mare	Parity	Gestation period.	Days	Sex of Foal
Rosie ...	<i>Secundi</i> :	317		Female
Rambler ...	<i>Primi</i> :	326		Male
Bounce (1941) ...	<i>Multi</i> :	331		Female
Rita ...	<i>Secundi</i> :	332		"
Rebecca ...	<i>Multi</i> :	340		Male
Bounce (1940) ...	"	343		"
Ruby ...	<i>Secundi</i> :	346		"
Mandolina ...	<i>Primi</i> :	349		Female
Bloom ...	<i>Multi</i> :	363		Male

The two mares with the shortest gestation periods ("Rosie"—317, "Rambler"—326) were mated once only, so no question of accepting service during pregnancy arises. Two others ("Rebecca"—340, "Mandolina"—349) also were served once only. In the remainder several matings occurred and the period is calculated from the date of the last service. There appeared to be no significant relationship between the duration of gestation and either the degree of parity or the sex of the offspring; in the latter connection it is interesting to observe, without drawing conclusions therefrom, that the males tended to be carried longer than the females.

All the foals were of normal development at birth and seven of them remained healthy, growing normally. The eighth, a colt out of the mare "Ruby," died 58½ hours after birth from *Bact. nephritidis equi* infection. Since the formation of the stud, some four years previously, only one other foal had been lost and that was due to dystocia. There had been no cases of joint-ill.

The First Stage of Labour.—It is customary to define the first stage as that of dilatation of the uterine cervix: strong uterine contractions commence and the foetal sacs are forced into and through the relaxed cervix thus dilating it. The best indication that this stage had commenced was the onset of "patchy" sweating, behind the elbows and over the flanks. This was seen in seven of nine cases, and commenced about four hours before the birth of the foal. In the early stages there were no obvious signs of pain: all the mares ate their food while some partook of it ravenously. There was no significant increase in the pulse rate (other than that which occurs in the late stages of pregnancy) and the body temperature, as has been previously stated, was subnormal. As the stage advanced the mare became restless and moved aimlessly around the loose-box. The tail was frequently raised or held to one side. Sometimes there was

swishing of the tail or slapping of it against the anus. As the end of the stage approached sweating increased and the mare became very restless. This was indicated by crouching, straddling of the hind legs, going down on the knees or breast and rising again, and glancing at the flank. The stage terminated with rupture of the allanto-chorionic membrane and the escape of allantoic fluid from the vulva. This was urine-like in appearance and never copious. It was impossible accurately to assess the quantity but the impression gained was that it never exceeded a gallon.

The Second Stage, namely, that of expulsion of the foetus. This stage was taken to commence either with the appearance of the amniotic sac at the vulva or the onset of straining, whichever occurred first. There was never much delay between them and in several cases they coincided. The onset of the stage was generally sudden. The amniotic sac appeared at the vulva, at first the size of an orange and a little later that of a coconut. As a rule the mare "went down" the moment straining commenced. She passed on to her side with limbs extended. In one case only ("Rosie") did straining occur while the mare was standing. The presence of the "water bag" at the vulva was quickly followed by the appearance in it of a digit. Straining efforts having commenced they recurred at fairly regular intervals until the foal was born. Each bout of straining comprised three or four powerful expulsive efforts followed by a period of rest. The rest-periods lasted from two to ten minutes, but most were of about three minutes. In two cases the mare regained the standing position during the straining period but quickly became recumbent again. In all cases the foal was born with the mare "stretched out" on her side, and in all cases also, the presentation of the foetus was anterior, in the dorsal position. It was particularly noticed that one fore-digit appeared in the amniotic sac 5 to 6 inches in advance of the other, and this position was maintained until the head, neck and fore-limbs were delivered. The point is a significant one, for it would appear that in normal parturition one elbow passes through the pelvic inlet before the other, and in this way Nature has provided that the foetus shall present the minimum obstruction at the pelvic inlet. The observation supports the teaching of Benesch (1938), who advises that when traction is applied for the delivery of a foetus in anterior presentation, it should be given to the fore-limbs alternately so that the elbows do not engage the maternal pelvic inlet simultaneously.

It was not possible accurately to assess the position of the foetus at the pelvic inlet, whether true dorsal or slightly oblique. During its delivery, the head was generally in the oblique position, but this was probably due to slight rotation of the cervical joints. In one case the head was born in the transverse position; the right cheek lying on the limbs. It is interesting to observe that the foetus does not come into direct contact with the maternal vagina during birth for the amnion forms a sheath through which it is delivered. It has

already been stated that up to the onset of parturition the vaginal mucous membrane was sticky. No doubt the allantoic fluid served to lubricate it for the passage of the amniotic sac, but the foetus itself only came into contact with the glistening amnion.

The foal commenced to breathe as soon as its nostrils came in contact with air, irrespective of whether or not it was completely born. In the cases of "Rosie," "Bloom," "Bounce" (1940), and "Mandolina," breathing commenced three to ten minutes before completion of birth. It would appear that normally the amniotic sac is ruptured by the pressure of the foetal digits, or by the weight of the foetal head and chest, or by the trunk when it "flops" out of the vagina. The limits of displacement of the amniotic sac will be governed chiefly by the length of the allantoic portion of the umbilical cord and the degree to which it is stretched without rupture. In those cases in which the allantoic umbilical cord was subsequently measured it was found to vary from 8 to 17 inches (to these figures must be added the 2 to 3 inches attached to the foal's belly). In one case ("Rita") the foetus was completely born with the amniotic sac still unruptured. It is probable in this case that had the sac not been torn by an attendant, movement of the foetus would have served to rupture it, for it is likely that there is a wide margin between the birth of the foetus and the separation of the placenta. In this particular case the placenta was not expelled until two hours later. There is a possibility, however, that a foetus born in its amniotic sac may succumb, particularly when delivery is quickly followed by separation of the allantois-chorion (see "Rosie," Table III).

TABLE III

Mare	Parity	Duration of Labour		
		1st Stage Hours	2nd Stage Minutes	3rd Stage Hours
Rosie ...	<i>Secundi :</i>	4	70	$\frac{1}{8}$
Bloom ...	<i>Multi :</i>	Unknown	50	11
Bounce (1940) ...	"	4	15	$\frac{1}{8}$
Mandolina ...	<i>Primi :</i>	2+	15	2-7
Rita ...	<i>Secundi :</i>	1+	23	2
Bounce (1941) ...	<i>Multi :</i>	2 $\frac{1}{2}$	9	3
Ruby ...	<i>Secundi :</i>	2-3	10	10 $\frac{1}{2}$
Rambler ...	<i>Primi :</i>	Unknown	30	14-21

Straining ceased with delivery of the foetal hips. The hind limbs remained in the maternal vagina and were withdrawn later, either as the result of movement of the foal or the mare rising from the ground. In all cases the umbilical cord was intact when the foal was born. Its subsequent rupture, 2 to 3 inches beneath the foal's belly, resulted from movement either of the mare or the foal.

It will be seen from Table III that this stage lasted from 9 to 70 minutes. In six cases it was 30 minutes or less ; in one, 50 minutes ;

and, in the longest, 70 minutes. In the two exceptionally long cases the delay occurred in the early part of the stage, that is until the time when the head was forced through the vulva. Much greater effort was necessary to reach this point in these two cases than in the remainder. Facilities for weighing the foals at birth were not available, but they were all well developed and of approximately the same size.

Expulsion of the foal was for the mare an enormous effort, and after its delivery she lay on her side exhausted. The duration of this continued recumbency varied, in five cases, from 12 to 30 minutes. An exception was the multiparous mare "Bounce." Her foaling was observed in 1940 and 1941. On the first occasion she expelled the foal in 15 minutes and rested for three minutes only, and on the second expulsion occupied nine minutes and the mare rose immediately afterwards.

The Third Stage—the period elapsing between the birth of the foetus and the expulsion of the foetal membranes.

It is generally accepted that in the mare expulsion of the placenta occurs quickly after the birth of the foal. According to Fleming-Craig (1930) separation takes place very rapidly due to the disseminated nature of the placenta and the slight adherence of the villi, and retention is exceedingly rare. Williams makes no definite statement regarding the mare but mentions that the placenta ordinarily drops away in the various species of domestic animals within 30 minutes after the expulsion of the foetus.

In the cases under review there were marked variations in the duration of this period. In one only did the membranes come away immediately after the birth of the foal; in the others they were retained for periods varying from $\frac{1}{2}$ to 14 to 21 hours (by this last time margin is meant that they were still retained when the mare was last seen at night but were expelled by morning).

It is the accepted veterinary teaching that retention of the placenta in the mare is a serious complication of parturition because of the rapidity with which metritis and toxæmia develop. Fleming-Craig state that so liable is the mare to septic infection that manual extraction of the membranes is always indicated when they are not expelled immediately after birth. The late Professor McQueen taught that if they have not been expelled twelve hours after the birth of the foal manual removal should be proceeded with. Nevertheless, the writer has discussed this matter many times with horse breeders and a number of occasions have been cited in which mares, after a normal parturition, did not expel the membranes for periods extending to 24 hours and yet came to no harm. The writer believes that he has encountered a number of cases in both the mare and the cow in which early interference was harmful. By manipulative separation the uterine epithelium becomes broken, the capillary loops in the crypts are opened and foci for the entry of bacteria are established. It is probable that the delay in expulsion of the membranes is due to some inertia of the uterus consequent upon the great effort it has been called

upon to perform, and it is reasonable to express the view that the organ should be given ample time to re-assert itself before interference is commenced. It was with these points in mind that it was decided not to consider interference within 24 hours in cases in which delivery of the foal was normal, but to try the use of post-pituitary extract. (It is admitted that each case must be considered separately when there has been prolonged, manipulative delivery resulting in the interior of the uterus already becoming a grossly infected focus.) The membranes were retained for an exceptionally long period in the mares "Bloom," 11 hours, "Ruby," $10\frac{1}{2}$ hours, and "Rambler," 14 to 21 hours. In the case of "Bloom" a dose of 10 c.c. of pituitary extract (Armour) was injected intramuscularly eight hours after birth of the foal. The onset of the drug's action became apparent in 15 minutes and it persisted for 30 minutes. There was severe abdominal pain and the mare went down on her side and strained forcibly. Flatus was voided but the membranes were uninfluenced, at least apparently. The latter were expelled two and a half hours later, by which time it was reasonable to assume that the drug's action had ceased. The post-puerperal period was normal: the mare's appetite was excellent, there was no significant rise in body temperature or vulval discharge, and the foal developed normally. "Rambler" was given 5 c.c. of pituitary extract ten and a half hours after delivery and a further 5 c.c. four hours later. Neither dose elicited any obvious response. The mare was eating, her temperature was 100.8° F., pulse rate 64, and the foal was sucking. At 21 hours (6 a.m. the following morning) the membranes were on the floor of the loose-box. The mare remained perfectly normal. The case of "Ruby" was somewhat different. The membranes were expelled ten and a half hours after a normal delivery, but the foal died $58\frac{1}{2}$ hours after birth from *Bact. nephritidis equi* infection. Autopsy, performed by the department of pathology of the Royal Veterinary College, revealed lesions in the kidneys and joints whereas the umbilical veins and arteries were normal. Swabs taken from the interior of the mare's uterus three days after parturition failed to reveal the above organism, as also did material from the placenta. The mare remained normal.

The Foetal Membranes.—The final observations concern the foetal membranes and placentae. It will be recalled that in the mare "Rosie" separation of the placenta occurred during the birth of the foetus, in fact part of the allantois-chorion was expelled surrounding the foal and the remainder came away when the mare rose. It is interesting to note that this was the case in which the second stage of labour was longest (70 minutes) and the speculation might be made that if expulsion had taken much longer death of the foetus would have ensued from separation of the placenta. The chorionic surface was outermost during expulsion. The membranes were examined in five other cases. In four, the allantois-chorion was completely inverted when expelled, that is with the allantoic surface

outermost. In these cases the intervals elapsing between birth and expulsion of the membranes were, respectively, two to seven, two, three, and ten and a half hours. The inference drawn is that separation of the placenta commenced at the apex of the gravid cornu, and under the influence of local uterine contraction, became inverted. Progressive contraction of the uterine musculature from before-backwards caused this inversion to proceed to completion. In the final case the membranes were retained 14 to 21 hours and expelled with the chorionic surface outermost. Assuming it to be normal for the placenta to be inverted during expulsion, except when it comes away with the foetus, a possible explanation of the last case is that owing to the delay in the onset of involution the placenta everywhere separated due to degenerative changes in the villi and fell into the lumen of the uterus.

TABLE IV

Mare	Period between Expulsion of the Foetus and the the Membranes	Surface of Allantois- chorion Outermost on Expulsion	Length of Umbilical Cord		Twists in the Umbilical Cord	
			Allantoic	Amniotic	Allantoic	Amniotic
Rosie ...	Immediately	Chorionic	—	—	—	—
Mandolina	2-7 hours	Allantoic	—	—	None	None
Rita ...	2 "	"	10 in.	6 in.	1	3
Bounce (1941)	3 "	"	14 "	14 "	1½	1
Ruby ...	10½ "	"	8 "	20 "	None	None
Rambler...	14-21 "	Chorionic	17 "	10 "	"	"

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TUBERCULOSIS OF THE BOVINE UDDER*

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INTRODUCTION

TUBERCULOSIS of the bovine udder is of great importance to the veterinary profession on account of its connection with both milk hygiene and meat inspection. The present work has been done in the hope that it will help to some extent in the diagnosis, both ante-mortem and post-mortem, of this condition.

The available literature dealing with tuberculosis of the udder is not large and reference to it shows some discrepancy of view as to its incidence, pathogenesis and morbid anatomy. M'Fadyean (1917), although discussing udder tuberculosis at some length, gives no indication as to its frequency in this country. According to Gofton (1926), abattoir statistics give the incidence as 1 to 2 per cent. of tuberculous cattle and later (1931) he states that 45 per cent. of the cases of udder tuberculosis occur in cattle in which the disease has not generalised. Nieberle (1930), in his work on tuberculosis in cattle in Germany, states that the udder is the organ most commonly affected after the lungs, while Joest and Kracht-Palejeff (quoted by Hutyra and Marek) state that 25 per cent. of cases of generalised tuberculosis show udder lesions. According to Savage (1929), "veterinary data indicated that about 40 per cent. of cows in Great Britain were affected with tuberculosis, the great majority of these being non-infective to the milk supply. The author is of the opinion that probably less than 1 per cent. of infected cows in Great Britain developed 'open' tuberculosis."

It is generally accepted that infection of the udder is by the blood stream (Nieberle, Sven Wall and Ostertag). M'Fadyean, after reviewing the literature, thinks that this view is erroneous and that the udder is usually infected from the lymph stream, giving as his reasons that the disease is often confined to one quarter, usually a hind quarter, that the lesions are rarely discrete tubercles, that the upper part of the quarter is affected first and that the supramammary lymph glands are usually involved before the udder.

As regards the morbid anatomy of the tuberculous lesions, M'Fadyean (1917) stresses the fact that tuberculous mastitis is not a nodular lesion. He states that he has never seen a case where the lesions took the form of sharply defined nodules or tubercles embedded in remains of the normal tissue of the udder, and remarks that these lesions must be very rare. Macroscopically the lesion can be diagnosed by the abnormal solidity and firmness of the quarter and by the exaggerated appearance of lobulation. Later, the oldest parts may show complete necrosis, caseation and calcification. Microscopically, he divides the lesion into three stages: (a) The stage of cellular infiltration between the acini of the lobules; (b) the stage when the infiltration of cells obliterates the normal histological features of the lobules, with giant cell formation; (c) the stage of necrosis and caseation

*This investigation was undertaken during the tenure of a Centenary Fellowship of the Royal Dick Veterinary College, 1941-42.

and finally calcification. Histologically, he stresses the fact that at no stage can the lesion be resolved into typical "tubercles," and even if these occur occasionally such groupings are highly exceptional and probably quite accidental.

Probably the most complete account of the pathology of udder tuberculosis has been given by Nieberle (1932). He divides the lesion into the following three distinct types:—

(a) Disseminated miliary tuberculosis, which develops as an early generalisation, is manifested by the formation of "lardaceous" and at a later stage caseous or calcified typical tubercles. These are always situated within the lobules and are usually aggregated in certain areas. The lymph glands are always affected and contain tubercles or larger caseous foci. This type of lesion is rare, since generalised tuberculosis usually occurs in an early period of life before the udder is fully developed and in that stage it is not readily infected.

(b) Chronic udder tuberculosis probably accounts for 80 to 90 per cent. of all cases of udder tubercle. Any statements, however, of its frequency of occurrence from naked eye examination are unreliable, due to the non-characteristic appearance. It is manifested by increased volume, tough and compact consistence and by the appearance of excessive lobulation on the cut surface, the latter having a lardaceous, translucent appearance.

Microscopically, the lesion is essentially productive and tuberculous granulation tissue with epithelioid cells and giant cells invade the lobules, destroying the acini. This infiltration, however, halts at the lobule edge. A caseous galactophoritis is present. The supramammary lymph glands are almost always free of tuberculous foci in this type, but occasionally show a simple lymphadenitis.

As to the pathogenesis of this type of lesion, Nieberle states that the tubercle bacilli reach the lobules from the blood stream and are there taken up and, like all chronic organ tuberculosis, the spread is intracanalicular.

(c) Caseous tuberculous mastitis is characterised, naked eye, by irregular areas of dry caseation, with haemorrhagic spots and patches, which are not limited by lobule boundaries. Microscopically, the nature of the lesion is essentially exudative. The cellular and fibrinous exudate quickly diffuses throughout large areas of the udder, breaking through interlobular septa. The affected areas then quickly become caseous. Hyperaemia is well marked. These diffuse lesions begin as small foci in a lobule, which spread and coalesce involving the whole lobule, and finally there is breakdown of interlobular septa so that many lobules coalesce. The supramammary lymph glands are always caseous with this type of lesion.

My own observations on udder tuberculosis, while concordant in many respects with those of Nieberle, reveal certain differences in detail which will appear in the account below.

ORIGINAL WORK

The work undertaken was the examination, both gross and microscopic, of tuberculosis in the udder of the cow. Udders were taken from animals which, on slaughter, were found to be affected with fairly widespread tuberculosis. A pathological examination was carried out on 27 such udders and of these 12 were found to contain tuberculous lesions.

In every udder examined, whether tuberculous or non-tuberculous, the minimum examination comprised the following: (a) Detailed naked eye examination of both halves of the udder, by means of multiple slicings of the organ. (b) Smears from the cut surface of all quarters; stained by the Ziehl-Neelsen technique and examined for tubercle bacilli. (c) Similar examination, naked eye, of the supramammary lymph glands was carried out. (d) Tissue was taken from two large areas in each quarter of the udder. In most of the cases, however, many additional blocks were taken from all regions, particularly at various levels above the ampulla, so as to trace the changes in the larger milk ducts.

At least six blocks were taken from each of the two areas and examined by the following staining methods. (a) Haematoxylin and Eosin. (b) Mallory's connective tissue stain. (c) Ziehl-Neelsen's stain for acid-fast organisms. French's elastica stain was used in many sections, but not in all.

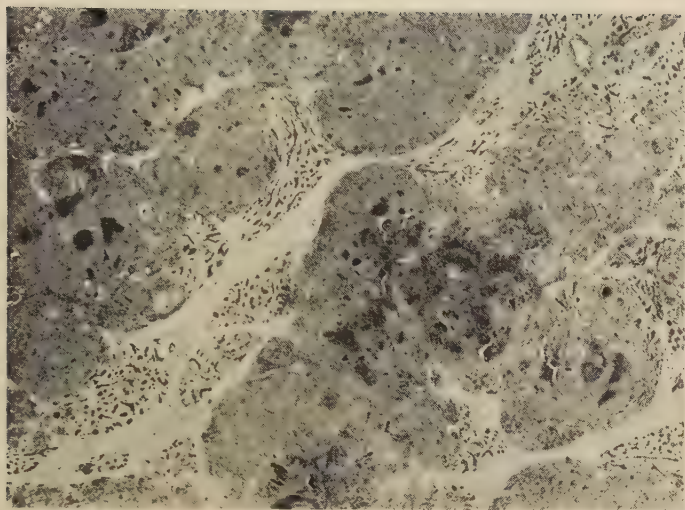
It was found that tuberculous udder lesions could be divided by means of macroscopic and microscopic examination into the following distinct types.

TYPE I. *Acute Caseating* (Cases 1 and 2.)

Naked eye: There was present an extensive and widespread destruction of lobular tissue, with large areas of haemorrhagic necrosis, in which there might be no trace of calcification. Softenings occurred throughout the affected quarters with the formation of abscesses. Occasionally massive calcification was found. The supramammary lymph glands in one out of the two cases showed naked eye lesions of tuberculosis.

Histology: Microscopical examination showed a complete breakdown of the normal gland substance in large areas. The lobules were no longer distinguishable individually since the tuberculous process had transgressed and destroyed the interlobular connective tissue septa. Groups of affected lobules were, however, still separated so that the gland was apparently made up of very large lobules separated by connective tissue. Each of these areas was made up, not of acini, but of necrotic and caseous material surrounded by a zone of tuberculous granulation tissue. Blood vessels were very numerous and haemorrhagic areas were commonly seen. Remnants of invaded lobules occurred here and there between the lesions (Fig. 1).

In less affected areas the appearances, while of the same nature as described above, were less advanced and were explanatory of the evolution of the process. Rapidly proliferative and caseating lesions were seen spreading from focal centres of tuberculosis in the individual lobule. The lesions reached sometimes halfway, sometimes to the edges of the lobule, while still remaining restricted to the single lobule. Here and there affected lobules occurred in groups and the tuberculous process was becoming confluent, so as to produce the large caseating areas first described.



ACUTE CASEATING TYPE

FIG. 1. Udder tissue showing large areas of haemorrhagic necrosis and caseation.

H. and E. $\times 7\frac{1}{2}$

CHRONIC DIFFUSE GRANULOMA TYPE

FIG. 2. Udder lobule showing replacement of normal tissue with foci of tuberculous granulation tissue and numerous giant cells. H. and E. $\times 50$

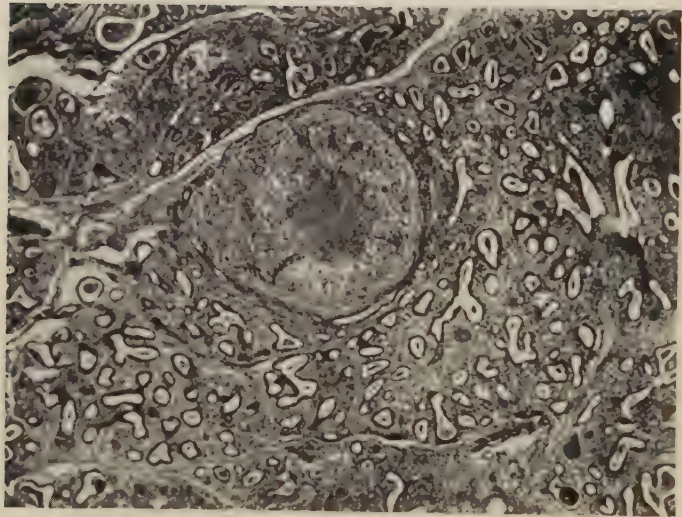
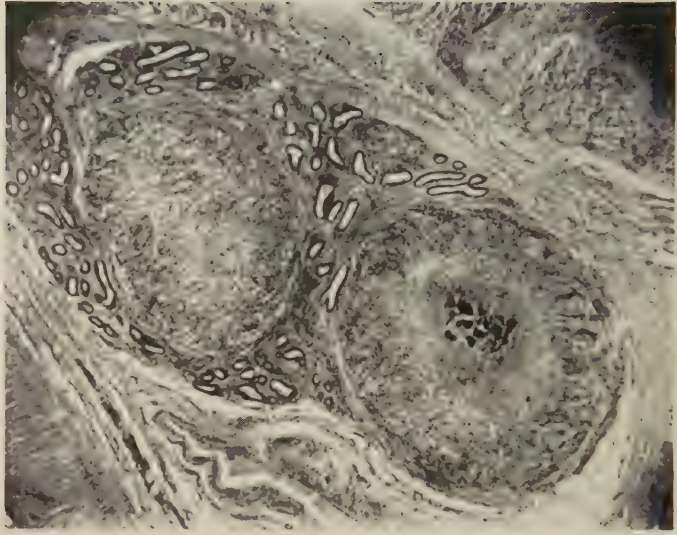


CHRONIC DIFFUSE GRANULOMA TYPE

FIG. 3. Udder tissue showing complete involvement of lobules with tuberculous granulation tissue but with no breakdown of interlobular tissue. H. and E. $\times 15$

CHRONIC NODULAR TYPE

FIG. 4. Caseous and calcified tubercles in an udder lobule.
H. and E. $\times 30$

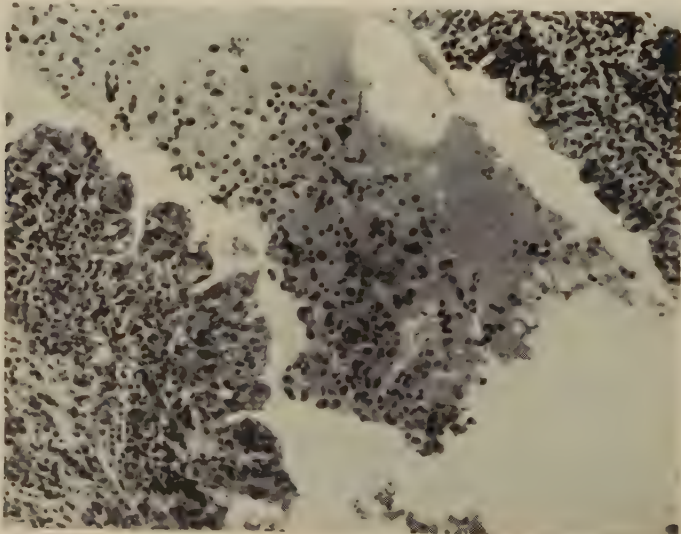


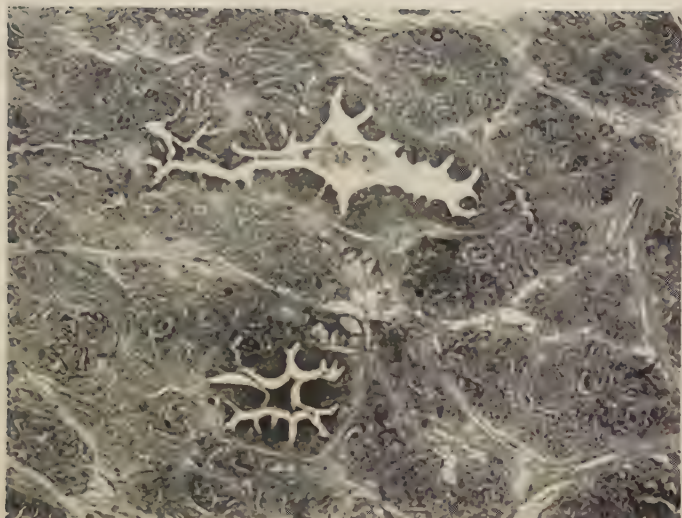
CHRONIC NODULAR TYPE

FIG. 5. Udder tissue showing a chronic encapsulated tuberculous lesion.
H. and E. $\times 30$

CHRONIC NODULAR TYPE

FIG. 6. Terminal interlobular duct showing papilliform projections of the epithelium due to tuberculous infection.
H. and E. $\times 150$

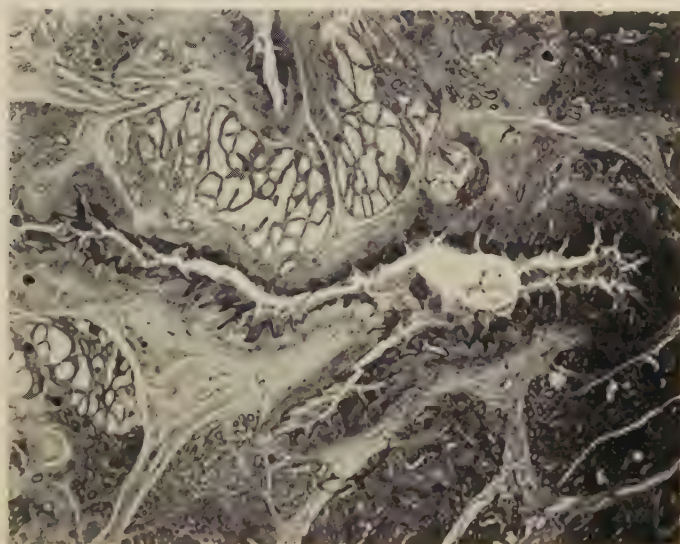




DIFFUSE TUBERCULOUS
GALACTOPHORITIS

WITHOUT NODULES TYPE

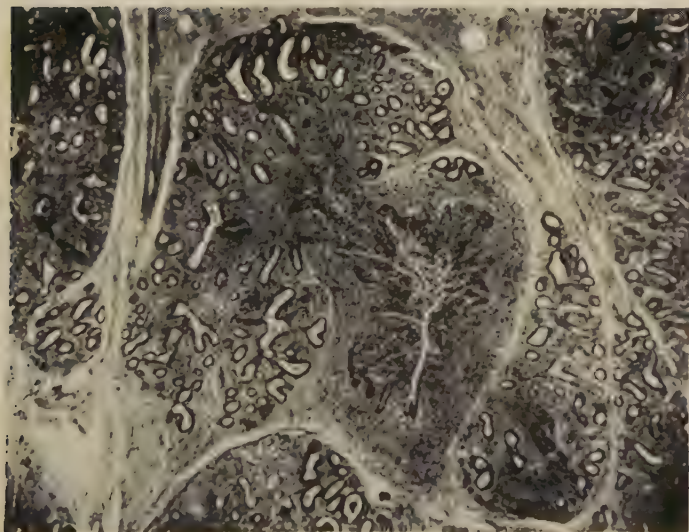
FIG. 7. Udder tissue showing tuberculous infection of the interlobular ducts. H. and E. $\times 15$



DIFFUSE TUBERCULOUS
GALACTOPHORITIS

WITHOUT NODULES TYPE

FIG. 8. Tuberculous change in an interlobular duct. H. and E. $\times 15$



DIFFUSE TUBERCULOUS
GALACTOPHORITIS

WITHOUT NODULES TYPE

FIG. 9. Tuberculous infection of a terminal interlobular duct with spread of infection to the intra-lobular ducts.

H. and E. $\times 30$



The interlobular ducts showed important changes. Where there were extensive lesions involving groups of lobules, the interlobular ducts were completely destroyed with the surrounding lobules. On the other hand, interlobular ducts, which were free of the large masses, exhibited a chronic tuberculous lesion; their walls were thickened by granulation tissue formation and, while focal tubercles were not identifiable, the process was demonstrated to be tuberculous by the presence of acid-fast bacilli.

In Case 1, where massive calcification of the udder had occurred, the supramammary lymph glands contained large calcified and caseous nodules almost entirely replacing the lymphoid tissue. In Case 2, the supramammary lymph glands showed no evidence of tuberculosis. They did, however, show a well marked chronic lymphadenitis of a non-specific character.

TYPE 2. *Chronic Diffuse Granuloma.* (Cases 3 and 4.)

Naked eye: The udder tissue, instead of having the usual flaccid glandular character, was solid and stiff, while the lobulated structure was greatly exaggerated so that the lobules projected from the surrounding interlobular connective tissue. The colour of the whole surface was chalky white instead of the normal pinkish-white. The surface was dry and there was no oozing of milk. The supramammary lymph glands showed no lesions of tuberculosis.

Microscopic examination: The lobular structure of the udder was maintained and the interlobular fibrous tissue, although thinned out, was not encroached upon. Almost every lobule in the involved quarter was invaded by tuberculosis. In rare instances an isolated lobule of fairly normal lactating type was seen or only one or two small tuberculous follicles were present in it. In the majority, however, the acinar structures were completely destroyed or only a few partially atrophied ones survived. The lobule area was then replaced by aggregations of tuberculous follicles with many giant cells; frequently these follicles had diffuse edges and were confluent, so that the focal appearance was obscured (Figs. 2 and 3). In occasional lobules the central part, which would involve the lobular duct, had broken down to caseation.

The interlobular ducts had a very striking appearance. The duct walls were greatly thickened to 150μ or more (as compared to the normal 20μ), being invaded by rather fibrous tuberculous granulation tissue. The lumen was thereby considerably diminished. The epithelial lining was usually completely destroyed, but in some instances persisted as a much altered layer of flattened squamous cells.

Supramammary lymph gland: Large secondary lymph nodules were present. The lymph sinuses showed some endothelial desquamation. Scattered through the peripheral part of the supramammary lymph gland were small tuberculous lesions ($30-60\mu$) consisting of small follicles of epithelioid cells, lymphocytes and plasma cells,

along with one or two giant cells. These nodules were very numerous, as many as 20 being present in one section. Acid-fast organisms were found, but were few in number.

TYPE 3. Chronic Nodular. (Cases 5, 6, 7, 8, 9 and 9A.)

Naked eye: The cut surface showed nodules 2-10 mm. in diameter projecting from the groundwork of the udder; these were whitish-yellow in colour and were obviously gritty when cut with the knife. They were unevenly scattered over the cut surface, being more numerous in some areas than others. In other cases the lesion, although similar, differed in that the nodules were not gritty, being either yellow and caseous or pale, tough and fibrous. Except in the case where the nodules were fibrous, the groundwork had the features of a non-lactating udder.

The supramammary lymph glands showed extensive caseous and calcified nodules when the udder showed calcification. In the udder with fibrous nodules, no lesions could be found in the supramammary lymph glands. In the two udders which had caseating nodules the supramammary lymph glands in one case showed naked eye lesions of tuberculosis.

Microscopically, the normal lobular character of the udder was maintained but numerous tuberculous lesions were present. The lesions in each case were confined to single lobules and there was no encroachment on the interlobular connective tissue. The normal structure of the morbid lobule was completely obliterated by the tuberculous reaction; there was a peripheral zone of tuberculous granulation tissue surrounding a caseous area with central calcification. At the periphery of a few lobules surviving acini could be seen. Some of the larger lobules showed two, three or four advanced lesions (Fig. 4). In Case 9 the lobule lesion was caseous without calcification. In Case 5 the lesion was dense and fibrous with a thick wall of connective tissue (Fig. 5).

The interlobular ducts were almost universally tuberculous. Their lumina were dilated and filled with a cellular exudate, neutrophils being present in large numbers as well as lymphocytes and large macrophages. The lining epithelium was proliferating and was many cells deep and often thrown up into papilliform projections (Fig. 6). Squamous metaplasia was also seen in places. The duct wall was greatly increased in thickness by tuberculous granulation tissue and fibrous overgrowth.

Supramammary lymph gland: Microscopic examination of the supramammary lymph glands showed very little remaining lymphatic tissue. The normal gland substance was almost completely replaced by calcified tuberculous nodules (1-10 mm.). In Case 5, however, where the nodules in the udder were fleshy and fibrous, no tubercles could be found in the lymph glands even microscopically. In Cases 7 and 9, where the nodules in the udder were caseous, the supramammary lymph glands in the former showed no lesions microscopically, while in the latter there was a chronic non-specific lymphadenitis.

TYPE 4. *Diffuse Tuberculous Galactophoritis without Nodules.* (Cases 10 and 11.)

Naked eye: The affected quarters were rather fibrous and compact, lobulation being demarcated by the well developed connective tissue stroma. The general colour was a pale pink. Scattered over the surface were groups of lobules which were yellowish in colour, suggesting early stages of caseation. Unlike tuberculous nodules they did not project from the cut surface.

Microscopically, the udder tissue retained its normal lobular character. Many of the lobules were in the regressive phase after lactation, while others, fewer in number, were still lactating. No old tuberculous lesions could be found in the lobules. The outstanding feature in these cases was the pathological change in the interlobular milk ducts (Figs. 7 and 8). These ducts were greatly dilated and thick-walled with the enlarged lumina filled with an inflammatory exudate, neutrophils, lymphocytes and monocytes and occasionally large masses of keratin. The epithelial lining of the ducts frequently showed metaplastic change from the normal low columnar to the stratified squamous type. Various stages in this metaplasia were seen; there was either a heaping up of columnar cells or a very definite keratinised squamous metaplasia. The walls were greatly thickened (500μ) and showed chronic inflammatory changes consisting of fibrous granulation tissue massively infiltrated with round cells but only an occasional tubercle. On tracing out many of the tributary ducts of these large interlobular ducts to the corresponding lobules, by serial section, the inflammatory change could still be seen to be present. In the smaller ducts, however, the lumina were occluded by cellular debris. Examination of the lobules feeding the occluded ducts showed them to be in the non-secreting phase. Tuberculous lesions were found in these lobules, but were restricted to the main lobular ducts, and the tuberculous process could be traced down in continuity to one of the larger interlobular ducts (Fig. 9). These intralobular lesions were all of much more recent character than those in the interlobular ducts. The majority of the lobules were affected as above, but occasional lobules were met with whose ducts, although emptying into large infected interlobular ducts, were themselves non-infected and consequently non-occluded. These lobules were in the stage of lactation. This was especially seen around the very large collecting ducts.

Supramammary lymph gland: Examination showed occasional microscopical tubercles just beneath or within the subcapsular sinus. The gland as a whole showed a chronic lymphadenitis of non-specific character.

In one case a careful and thorough examination of the udder revealed no tuberculous lesion of any type, although the supramammary lymph glands showed naked eye lesions of caseating tuberculosis. It is, however, difficult to assess the importance of this observation since tuberculous lesions can be easily missed.

DISCUSSION

Incidence : Of the 27 udders examined from tuberculous cattle retained, but not necessarily totally condemned, in the detention room of an abattoir, 12 were found to be tuberculous, while in one case tuberculous lesions were found only in the supramammary lymph glands. It is appreciated that the number examined is not large, but the udders were accepted without selection, so that the results are considered to have some significance as regards frequency of incidence, although cows in the abattoir could not represent a cross section of tuberculous cows in the country. As can be seen, the percentage figure of these tuberculous cattle with udder lesions in this investigation is 44 per cent. This is considerably higher than the figure generally quoted in Great Britain, but is nearer to the figure mentioned by Joest in Germany (25 per cent.). Nieberle, although not stating definite figures, gives the udder as the organ most frequently affected after the lungs. In assessing the significance of my figures, it should be kept in mind that they were arrived at by careful and thorough histological examination as well as by naked eye examination. In this connection it is important to stress the fact that three cases would have escaped my recognition if only naked eye examination had been relied upon, and three more cases were, on naked eye examination only, suspected of being tuberculous and could easily have escaped detection in the ordinary methods of meat inspection. It is essential that more extensive pathological investigation be carried out to establish the true incidence of udder tuberculosis in this country. This need not necessarily involve very elaborate procedures. The results suggest that for the establishment of a positive diagnosis in a large series of cases, a thorough examination of suitably taken films for tubercle bacilli would be adequate ; according to Nieberle, a frozen section stained by haemalum and eosin gives a satisfactory diagnosis.

The present work is by no means complete but it at least shows that our knowledge of a very important disease process is grossly inadequate and that elucidation of the many problems which have arisen with this introductory work is essential.

Distribution of the lesions. The distribution of the lesions in the udder varies widely ; in some cases all four quarters were affected with tuberculosis, whereas in other cases the lesions were confined to two quarters. The following table (Table I) shows the distribution in the cases examined, along with the presence or absence of tuberculous lesions in the supramammary lymph glands.

Nature of lesions. It has been found that tuberculous lesions in the udder show considerable differences in type, and this finding is in general agreement with that of Nieberle. An important practical aspect of this variation in different cases is its bearing on the possibility of physical diagnosis during life. The acute spreading types of lesions (Type 1) and the more chronic but widespread granulomatous lesion (Type 2) probably present little trouble, but other

types (Types 3 and 4) must present a very difficult or impossible task in recognition during life.

The different types of involvement which have been described represent, in the main, different degrees of acuteness and chronicity in the nature of the lesion. There has, of course, been no possibility of obtaining in any one case a correlation between the end result observed and the clinical evolution of the disease, but the histological changes appear to give reliable evidence as to relative rate of progress of lesions. The acute caseating types show evidence of rapid progress and may be regarded as indicative of low resistance in the animal; whether this is dependent on a local allergy or on a more general condition of ill-health is not known. The more chronic lesions

TABLE I
DISTRIBUTION OF LESIONS

	Case No.	L.F.Q.	R.F.Q.	L.H.Q.	R.H.Q.	Supramammary lymph gland —naked eye	Supramammary lymph gland —microscopic
Group I	1	+	+	+	+	+	+
	2	early + (L)	early + (L)	+	+	—	—
Group II	3	+	+	+	+	—	+
	4	+	+	+	+	—	+
Group III	5	+	+	+	+	—	—
	6	+	+	+	+	+	+
	8	+	+	+	+	+	+
	9A	+	+	+	+	+	+
	7	+	+	—	—	—	—
	9	—	—	+	+	—	+
Group IV	10	+	+	—	—	—	+
	11	—	—	+	+	—	+
	12	—	—	—	—	+	+

L = Large numbers of lactating lobules.

1 = Occasional lactating lobules.

(Type 3) point with certainty to a duration of months, possibly years, thus suggesting some degree of immunity which results in a slow progress of the lesion, but which is insufficient to cause destruction of the tubercle bacilli. This is a common finding both in bovine and human tuberculosis in general.

Method of infection. The lesions have in all cases been fairly advanced, so that direct evidence as to the method of initial infection of the udder has not been obtained, but some deductions are possible from the facts observed. In the first place, the udder lesions have been found in cattle with fairly extensive internal tuberculosis and it appears fairly certain that the udder, as a peripheral organ, has been involved secondarily, the disease having spread from elsewhere. The possible routes would be by lymphatics or by the blood stream. My findings do not support the theory that the lymphatic vessels carry infection to the udder: the supramammary lymph glands

frequently showed no naked eye or microscopic lesions of tuberculosis, even when the udder was extensively invaded, while in other cases only microscopic tubercles were present in the supramammary lymph gland. In addition, if infection were by the lymph stream one would expect the udder to show always a characteristic pathological picture with older lesions nearest to the lymph gland, but this, in contradistinction to M'Fadyean, I have not found. It has also been shown that there may be very extensive involvement of ducts and lobules throughout a quarter without there being any encroachment on perilobular connective tissue in which lymphatic vessels lie. For this reason I believe that the initial settling of bacilli is from the blood stream with subsequent extension in the udder by the ducts. (The process of blood stream infection of an organ with later extension in the organ is often seen in the human body, *e.g.*, in kidney.) In this event it would appear that the organisms in a tuberculous bacillaemia may lodge only in two quarters (possibly one) and spread extensively there, while other quarters remain healthy and secreting. In Type 3 (chronic nodular) numerous old but not obsolete lesions were widely scattered and these could represent multiple sites of initial blood stream infection, but in Types 2 (chronic diffuse granuloma) and 4 (diffuse tuberculous galactophoritis without nodules) no older focal lesions in lobules were observed which could be accounted for in this way.

One of the most striking features in all cases has been the tuberculous invasion of the duct system. The existence of this was overlooked in the first few cases examined, owing to the rather non-specific appearance of the changes in the ducts. Later, when occasional tubercle follicles in the duct walls and the invariable presence of acid-fast bacilli had been noted, the true nature of the duct inflammation became apparent and re-examination of all material led to the finding of this lesion in every case. The extensive involvement of the duct system seems to point with certainty to the overwhelming part played by it in the spread of bacilli in the udder. In cases where old nodular lesions occur in scattered lobules (Type 3) the whole duct system is also infected and there is thus the possibility of the duct infection having arisen secondarily from primary lobule lesions. On the other hand, two cases (Type 4) show the same universal duct involvement with no old lobule lesions, while there is evidence of quite recent and early spread back into the lobular ducts and acini. The exact site of the first lodgement of the bacilli thus remains uncertain, but a general view of the whole of the material suggests that it is high up in the duct system and that the first tuberculous lesion is in the duct wall, the lobules being affected secondarily to the ducts. One most suggestive feature is that the duct system involvement may affect only two quarters while two escape. Such cases leave no doubt that the process extends by some local mechanism in the tissue and that the line of transport of tubercle bacilli is the lactiferous ducts. The fact of existence of a lumen in which infection

can spread is no doubt important, but the possibility has also to be considered that the tissue of the ducts is less resistant to bacterial invasion than that of the lobules.

It is considered that the most important histological finding in all cases is this extensive involvement of the duct system. It is of the greatest practical importance to recognise that in every case examined in this work, no matter whether the lesions were old and partially healed or young and active, the ducts show a universal tuberculous inflammation in the affected quarter. In other words, every tuberculous udder is an open case of tuberculosis and as such is a possible danger to public health. A further interesting feature is that the tuberculous lesion in the terminal interlobular ducts leads frequently to a blocking of this duct, so that the milk is prevented from passing from the secreting lobule to the larger ducts. This appears to cause an atrophy of the secreting acini which enter the resting stage. It is deduced that this duct blockage with the resultant atrophy of the lobules leads to the progressive "drying up" of the udder rather than tuberculous destruction of secreting tissue. It is, however, important to note that even where much of the quarter is non-lactating, a few lobules immediately surrounding the larger ducts are still lactating so that there must be a continuous risk of milk contamination. This is the more important when one realises that other quarters of the same udder are sometimes still in full lactation, so that there is a continuous danger that whatever little milk can be withdrawn from the infected quarter may reach the milk pail.

SUMMARY

1. Twenty-seven udders from tuberculous cows were examined ; of these 12 were affected with tuberculosis, while one showed lesions only in the supramammary lymph glands.

2. The presence of tuberculosis in the udder has been ascertained by naked eye examination more frequently than by manual examination and still more frequently by histological examination than by naked eye examination.

3. The presence or absence of naked eye lesions of tuberculosis in the supramammary lymph glands gives no indication as to the involvement of the udder.

4. The distribution of the lesions in the udder has been described. Tuberculosis does not necessarily invade all four quarters of the udder, nor is there any apparent relationship between the type of lesion and the quarters infected.

5. Four types of tuberculosis of the udder have been described both naked eye and microscopically. The type apparently depends on the resistance of the animal and not on the mode of infection. Tubercle bacilli have been found in all the cases described.

6. In all cases, whether chronic or more active, there has been an extensive tuberculosis of the duct system. In other words, all

cases of tuberculosis in the udder are open lesions. The inflammatory change in the ducts is not very typically tuberculous histologically, but is characterised by the presence of the tubercle bacilli.

7. Even very advanced lesions of tuberculosis in the udder may leave some lobules lactating. It would therefore appear that the affected quarter can still secrete a little infected milk. This is even more important when it is realised that other quarters are still, in some cases, fully lactating and non-infected, so that the cow may still be in use as a milker.

8. The original infection of the udder by tuberculosis is presumed to be by the blood stream. Once infected, however, the infection is evidently spread by the duct system.

ACKNOWLEDGEMENTS

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FURTHER EVIDENCE ON THE SIGNIFICANCE OF VACUOLATED NERVE CELLS IN THE MEDULLA OBLONGATA OF SHEEP AFFECTED WITH SCRAPIE

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INTRODUCTION

THE presence of vacuolated nerve cells in the spinal cord and medulla oblongata of sheep affected with scrapie has been recorded by pathologists in Britain and on the Continent. The significance and specificity of this pathological change have been doubted by the majority of neuro-pathologists, largely because vacuolation of nerve cells occurs in a variety of other disease conditions, may be experimentally produced, may be due to artefact, and may be seen occasionally in apparently healthy animals. In the case of sheep affected with scrapie a diligent search has been made over a period of many years to discover some more specific, and therefore more significant, pathological change in the nervous or other tissues. Brownlee (1940) has reviewed the literature dealing with these investigations and, adding his own observations to the findings of others, has come to the conclusion that vacuolation of nerve cells in the medulla oblongata and/or spinal cord is constantly present in scrapie. He has suggested, however, that "the distribution of nerve cell systems in the sheep is not sufficiently well known to permit the inference that selective injury has been inflicted on the cells of any particular nerve cell group or groups."

This paper is an attempt to confirm Brownlee's finding that vacuolated nerve cells are found in all cases of scrapie, to describe the distribution of vacuolated cells in cases of scrapie, and to determine the degree of significance that can be attached to the finding of vacuolated nerve cells in the brain stem of the sheep.

METHOD OF EXAMINATION

All tissues were treated in the same way—fixed in 10 per cent. formol-saline, dehydrated through the alcohols, cleared and hardened in chloroform, and embedded in paraffin. Three staining methods were used—haematoxylin and eosin, Loyez's modification of Weigert's stain, and Mann's stain. One complete medulla from a case of scrapie was cut into 1,675 sections, and two others, also from clinical cases of the disease, were examined in 10th section and 20th section series respectively. In all other cases one to six sections cut at random through the medulla were examined.

PRESENCE OF VACUOLATED CELLS IN CASES OF SCRAPIE

Table I shows that of 75 clinical cases of scrapie vacuoles were found in cells in the medulla oblongata in every case. In the great majority of cases vacuoles were found easily. In two cases, however, three sections had to be examined before vacuoles were found, and in one case it was necessary to examine five sections. In contrast to this, no vacuolated cells were found in 58 apparently healthy sheep—comprising 52 in which two sections were examined and six in which six sections were examined—or in a varying number of sections from 35 sheep that had died from diseases other than scrapie or had been accidentally killed.

TABLE I
INCIDENCE OF NERVE-CELL VACUOLATION

<i>Clinical</i>	<i>Number of Sheep Examined</i>	<i>Vacuoles Present</i>	<i>Vacuoles Absent</i>
Sheep affected with scrapie and brought to Moredun before death	40	40	0
Dead sheep sent to Moredun with shepherd's diagnosis of scrapie	35	35	0
Totals	75	75	0
Apparently healthy sheep killed for examination	59	1	58
Sheep at Moredun dying from diseases other than scrapie	5	1*	4
Dead sheep sent to Moredun with shepherd's diagnosis as to cause of death:—			
Drowning	3	0	3
Braxy	9	0	9
Lambing trouble	3	0	3
Couped (overturned)	4	0	4
Liver fluke	2	0	2
Pine	12	3	9
Fits	1	0	1†
Totals	98	5	93

* Pregnancy toxæmia.

† Perivascular infiltration present.

Vacuolated cells were found, however, in one apparently healthy sheep and in four other sheep that had been killed or had died of diseases other than scrapie (assuming the shepherd's diagnosis to be correct).

Much has been written on the part played by artefact in the production of vacuoles in nerve cells. Vacuoles have been seen in diseases of sheep other than scrapie. However, a statistical test of the data summarised in Table I, using Yates' modification, gives $\chi^2 = 155$ and P much less than 0.001. A correlation between vacuolated nerve cells and scrapie can, therefore, hardly be doubted.

Whether or not all cases of scrapie show vacuoles is not known. Table I indicates that vacuoles were found in each of 75 cases of scrapie. Assuming that the true occurrence of vacuolated cells in scrapie is 94 per cent., then the standard deviation would be $\sqrt{75 \times 0.06 \times 0.94} = 2.06$. Twice the standard deviation = 4.12. Therefore, in 75 cases of scrapie the expected number of sheep showing vacuoles should not exceed $70.5 + 4.12 = 74.62$. Thus it is unlikely that in any larger sample there would be more than six cases out of every hundred in which vacuoles could not be found.

In view of the work of Andrew (1941) who, by starvation, produced vacuoles in nerve cells of the mouse, there is a possibility that the occurrence of vacuoles in sheep may be due, to a lesser or greater extent, to faulty nutrition. This suggestion receives some support from the fact that three of the non-scrapie sheep in which vacuoles were found were, according to the shepherd's diagnosis, affected with "pine." On the other hand, vacuolated cells have also been found in sheep that appeared to be in good bodily condition, although clinical symptoms were manifest.

Distribution of Vacuolated Cells in the Medulla Oblongata

In scrapie vacuolated cells occur in greatest concentration in the medulla oblongata, although they have also been found in the spinal cord, throughout the pons, and also in the mid-brain. We have not been able to demonstrate them in the cerebral cortex or cerebellum.

Tables II and III have been prepared by examination of serial sections from two medullas. The term "cell-group" has been used to designate a transverse section of a nucleus (*e.g.*, hypoglossal), or other recognised collection of cells (*e.g.*, lateral reticular formation). Therefore in the case of paired nuclei there will be two cell-groups in one transverse section through the medulla, *i.e.*, one cell-group on each side of the mid-line. The total number of vacuolated cells counted is given, and also the percentage of cell groups containing vacuoles in varying numbers. The size and position of these nuclei has been described in a previous article (Pattison and Holman, 1943).

Examination of these tables suggests that although vacuolated cells have been found in all areas of the medulla their concentration is not entirely haphazard, and that there may be areas of predilection in certain nuclei. Thus in the two medullas represented by the tables vacuoles were a great deal more common among cells of the reticular formation and medial vestibular and lateral cuneate nuclei than, say, among cells of the hypoglossal nucleus or inferior olive. Random sections from other scrapie sheep have confirmed this general plan of distribution.

Significance of Vacuolated Cells in the Medulla of the Sheep

It has been stated that vacuolated cells have been found in the medulla oblongata in every case of scrapie. It has also been stated that similarly vacuolated cells have been found in a comparatively

TABLE II
ANATOMICAL DISTRIBUTION OF NERVE-CELL VACUOLATION IN MEDULLA FROM SCRAPIE SHEEP No. 149

Anatomical Region	Number of Cell Groups Examined	Total Number of Vacuoles	No Vacuoles	Cell Groups (per cent.) containing:—					Over 15 Vacuoles
				1 Vacuole	2-5 Vacuoles	6-10 Vacuoles	11-15 Vacuoles		
Hypoglossal	710	29	97	2	1	—	—	—	
Gracilis	460	37	93	6	1	—	—	—	
Salivary	170	19	90	7	3	—	—	—	
Ambiguus	670	131	83	16	1	—	—	—	
Inferior olive	1,020	346	78	16	6	—	—	—	
Spinal trigeminal	2,220	1,669	61	22	16	1	—	—	
Ventral cochlear	290	224	60	23	16	1	—	—	
Facial	960	785	58	23	18	1	—	—	
Cuneatus	820	613	57	28	14	1	—	—	
Interfascicularis hypoglossi	500	419	54	27	19	—	—	—	
Rostral continuation of ventral horn cells	400	395	48	28	24	—	—	—	
Dorsal motor vagus	1,360	2,270	45	18	33	3	1	—	
Solitaris	630	762	41	29	29	1	—	—	
Praepositus and paramedianus	1,160	878	39	22	38	1	—	—	
Superior olive	290	396	35	31	33	1	—	—	
Abducens	220	301	34	32	34	—	—	—	
Lateral vestibular	620	1,257	23	23	51	3	—	—	
Lateral reticular formation	2,910	10,011	14	13	49	16	7	1	
Medial reticular formation	2,080	5,475	13	16	62	8	4	—	
Lat. cuneatus	790	3,671	8	6	40	42	1	—	
Medial vestibular	1,060	4,178	6	—	58	28	1	—	
Middle reticular formation	860	3,762	3	—	56	36	1	—	

TABLE III
ANATOMICAL DISTRIBUTION OF NERVE-CELL VACUOLATION IN MEDULLA FROM SCRAPIE SHEEP NO. 394

Anatomical Region	Number of Cell Groups Examined	Total Number of Vacuoles	No Vacuoles	Cell Groups (per cent.) containing:				
				1 Vacuole	2-5 Vacuoles	6-10 Vacuoles	11-15 Vacuoles	Over 15 Vacuoles
Hypoglossal	60	0	100	—	—	—	—	—
Gracilis ...	80	15	83	15	2	—	—	—
Salivary ...	...	...	...	...	...	...	...	...
Ambiguus	6	0	100	—	—	—	—	—
Inferior olive	120	5	97	2	1	—	—	—
Spinal trigeminal	100	19	83	16	1	—	—	—
Ventral cochlear	215	68	78	18	4	—	—	—
Facial ...	55	42	56	27	17	—	—	—
Cuneatus	80	9	91	8	1	—	—	—
Interfascicular hypoglossi	85	35	73	20	7	—	—	—
Rostral continuation of ventral horn cells	40	63	28	35	35	2	—	—
Dorsal motor vagus	100	1	99	—	—	—	—	—
Solitarius	110	64	69	19	11	1	—	—
Praepositus and paramedianus	95	56	66	21	13	—	—	—
Superior olive	95	33	76	19	5	—	—	—
Abducens	20	6	70	30	—	—	—	—
Lateral vestibular	20	2	90	2	—	—	—	—
Lateral reticular formation	55	42	60	22	18	—	—	—
Medial reticular formation	195	488	24	23	38	15	—	—
Lateral cuneatus	175	247	40	26	30	4	—	—
Medial vestibular	85	311	12	7	55	24	2	—
Middle reticular formation	120	355	21	20	37	20	2	—
	90	123	43	24	30	3	—	—

small number of sheep apparently unaffected by this disease. The finding of vacuolated cells, therefore, cannot be accepted as pathognomonic of the disease but clinical symptoms suggestive of scrapie followed by microscopic discovery of vacuolated nerve cells in the medulla oblongata probably constitute sufficient evidence on which to base a positive diagnosis of the disease.

DISCUSSION

We have shown that the presence of vacuolated nerve cells in scrapie is not due to artefact and that the distribution of such damaged cells varies in intensity in different nuclei. In many cases we have noted an apparently healthy cell containing well marked Nissl granules, clear nucleus and nucleolus, and normally stained cytoplasm containing a small vacuole, and other apparently healthy cells have been found scattered among cells distorted by vacuolation. In other cases, however, vacuolation has been superimposed on marked degeneration of practically all the nerve cells. We have been unable to stain the vacuoles by a variety of staining methods, including a glycogen stain and fat stains (unpublished), but in rare cases homogeneous granules staining pink with eosin have been observed within vacuoles.

The distribution of vacuolated cells suggests that one region of the medulla, by virtue of the nuclei it contains, is more likely than others to contain vacuoles in cases of scrapie, namely, the region just anterior to the calamus scriptorius. It is suggested, therefore, that sections for diagnosis should be cut through this region, and that more than one section should be examined.

SUMMARY

1. Of 75 sheep affected with clinical symptoms of scrapie all showed vacuolated nerve cells in the medulla oblongata. Statistical examination of this finding indicates that in further samples vacuoles would be expected in not less than 94 per cent. of cases.

2. Vacuolation occurs but rarely in sheep dying from other conditions.

3. The distribution of vacuolated cells in the medulla oblongata in scrapie is selective for certain groups of cells, notably the reticular formation and medial vestibular and lateral cuneate nuclei.

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A SEARCH FOR DIAGNOSTIC SYMPTOMS AND LESIONS IN A VARIETY OF SMALL EXPERIMENTAL ANIMALS AFTER ADMINISTRATION OF SWINE FEVER TISSUE SUSPENSIONS BY DIFFERENT ROUTES

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INTRODUCTION

THE diagnosis of swine fever, important in both the field and the laboratory, is at present based on the history, the *post-mortem* lesions and, where these are inconclusive as so often occurs in the early stages of an outbreak or in acute cases of the disease, on the results of the inoculation of pigs with tissue filtrates. Nevertheless, the expense involved in the purchase of pigs and their maintenance in suitably isolated accommodation makes this last method of diagnosis practicable in only a small proportion of cases.

The object of the present work was to discover whether infection could be transmitted to a small laboratory animal which would then be of practical value in the diagnosis of acute swine fever. Previous work has been done in this field of research but with conflicting results. In the present investigation a study was made in mice, hamsters, ferrets, white rats, guinea-pigs and rabbits, of any clinical symptoms following the administration of swine fever tissue suspension by various routes, and of the histological reaction in the brain after intracerebral inoculation. The latter procedure was suggested as a result of experiments on the survival of swine fever virus in the brains of small laboratory animals, experiments which were made as a preliminary to attempted intracerebral passage. They will be described in a later paper on that subject.

Examination of the literature dealing with previous work in this field demonstrated divergent results. Uhlenhuth and Haendel (1913) concluded from results of many experiments that various animal species including mice, rats, rabbits and guinea-pigs were insusceptible to the virus of swine fever. Stockman (1915) found that wild rats were unaffected by feeding with swine fever intestines. Swine fever tissue fluids inoculated intravenously into rabbits by Craig (1917) in volumes of up to 15.0 c.c. were without effect. Administration of swine fever virus by various routes to rabbits, guinea-pigs and rats by Ten Broeck (1918, 1920) gave negative results. The transmission of swine fever virus through rabbits and larger animals without apparent clinical signs was reported in the United States (1920). Böhmer (1925) and Brockmann (1926) stated that swine fever was not experimentally transmissible to the rabbit and Frohböse (1925) confirmed the insusceptibility of guinea-pigs to the virus, all three workers basing their conclusions on clinical signs and microscopical examination of blood films. Schubert (1928) reported that white mice and guinea-pigs, which had been given anti-swine blood guinea-pig or rabbit serum intraperitoneally, were unaffected by subcutaneous injections of highly virulent swine fever blood. Uhlenhuth and Geiger (1929), Graüb and Zschokke (1923) and Zen-Ruffinen (1922) found

that subdural injection of swine fever virus into rabbits and guinea-pigs and instillation on to the scarified cornea were without result. Transmission experiments by Jacotot (1937) in various mammals and birds, including guinea-pigs, rabbits and ferrets (pole-cats), showed that, although the swine fever virus could be recovered from the blood of these animals up to three to six days after injection, there was no clinical evidence of infection.

On the other hand, Ottolenghi (1913) stated that he found certain very virulent swine fever virus strains to be pathogenic for guinea-pigs by the subcutaneous and intraperitoneal routes and for rabbits by the intravenous route. Craig (1916) produced a fatal disease in four rabbits by the injection of 1.5 to 2.0 c.c. of swine fever blood and six out of eight pigs died of acute swine fever after injection with their tissue filtrate. Fekete (1928) reported that rabies-like symptoms were produced in rabbits, guinea-pigs, and mice by subdural injection of the brain tissue of swine fever pigs, but Uhlenhuth and his colleagues subsequently expressed the opinion that the swine fever virus strain employed had been contaminated with rabies virus. Krüger (1934) produced a fleeting temperature reaction by a single or repeated infection of blocked, splenectomised guinea-pigs with swine fever virus but did not establish the specificity of his results. In 1936, Le Chuiton, Mistral and Dubreuil described the successful passage of two swine fever virus strains in guinea-pigs by simultaneous intratesticular and intracerebral injection with the appearance of fever, dullness and loss of activity, which symptoms were maintained throughout subsequent passages. Moreover, they observed pulmonary and testicular lesions in later passage guinea-pigs. They also claimed that not only was the white rat susceptible to the swine fever virus but that the virus could be passed from rat to rat. Jacotot (1939) confirmed his previous results showing that while swine fever virus could be transmitted to various other animal species, it merely resided in the blood, causing a latent infection. He criticised the report of Le Chuiton and his colleagues (1936) that a febrile disease due to the swine fever virus could be transmitted from guinea-pig to guinea-pig, on the grounds that they had not satisfactorily demonstrated the presence of virus in the passage guinea-pig tissue used as the inoculum, and had ignored the possibility of intercurrent disease. In 1939, Vechiu produced a fatal disease in rabbits by a combination of intracerebral and intra-ocular or intraperitoneal injection of brain filtrate, cerebrospinal fluid and serum of swine fever pigs. The disease was characterised by nervous symptoms and various blood modifications, was transmitted in rabbits up to the 23rd passage by intracerebral and other routes and the passage rabbit virus was successfully transmitted intracerebrally to guinea-pigs and sheep, producing a fatal disease.

CLINICAL INVESTIGATIONS

General Procedure.—Appropriate doses of the supernatant liquid from 5 per cent. virulent desiccated swine fever spleen or brain suspension (after sedimentation) were administered orally, intradermally, subcutaneously, intraperitoneally, intranasally or intracerebrally to groups of six mice, white rats, rabbits and guinea-pigs. Control groups of equal size or half that number were given the same dose of supernatant liquid from 5 per cent. desiccated normal pig spleen suspension by corresponding routes. In some instances fresh whole blood, haemolysed blood and serum were used. The inoculum was proved to be bacteriologically sterile by aerobic and anaerobic

culture and virulent to a high titre for pigs. In the case of hamsters and ferrets, groups of three animals were used and only the intraperitoneal, intranasal and intracerebral routes were employed. Both test and control groups were kept under close observation for six weeks after the administration of a spleen suspension and in the case of white rats, ferrets, rabbits and guinea-pigs, the temperature of each animal was taken rectally each day for six days and then on the eighth and tenth days. The temperature charts of the test and control animals were compared at the end of that period for evidence of a significant reaction. Both test and control animals were killed after six to eight weeks and *post-mortem* examinations made. The technique adopted for intracerebral inoculation was similar to that employed by other workers and was carried out under full ether anaesthesia.

Clinical Results

A table is given of the results obtained in guinea-pigs to illustrate the methods employed for all the experimental animals.

Guinea-Pigs.—Of the 42 guinea-pigs, to which the supernatant liquid from swine fever desiccated spleen or brain suspension was administered by various routes, four died of intercurrent disease within the three weeks' period of observation. All other guinea-pigs remained without clinical signs of infection and the only lesions seen at autopsy after six weeks were those of intercurrent disease in five guinea-pigs.

Mice.—Seventy-two mice, to which swine fever virus was administered by various routes in the form of fresh whole blood, haemolysed blood and serum as well as the supernatant liquid from desiccated spleen or brain suspension, showed no significant difference from the control mice to which corresponding normal pig tissues had been given by the same routes, during observation for three weeks. All the organs appeared normal at autopsy after a further three weeks. Fifteen mice from both test and control groups, which showed dullness and inappetence with occasionally conjunctivitis, subsequently died of intercurrent disease.

Hamsters.—Of eleven hamsters injected by various routes with swine fever virus in the form of serum or the supernatant liquid from desiccated spleen suspension, only one showed dullness and inappetence, dying 13 days after intracerebral injection. No *post-mortem* lesions were observed in the organs and virus could not be recovered from the brain. The other ten hamsters showed no clinical signs of infection during three weeks' observation and no lesions were found in any organ at autopsy after a further four weeks.

Ferrets.—Of 16 ferrets injected by various routes with the supernatant liquid from desiccated or frozen swine fever spleen or fresh swine fever brain suspension, one showed a raised temperature with loss of appetite on the tenth day after intracerebral injection and died on the 18th day. Although meningeal adhesions were found at

CLINICAL RESULTS OF ADMINISTRATION OF SWINE FEVER TISSUE SUSPENSIONS TO GUINEA-PIGS

Number of Guinea-pigs.	Route.	Dose in c.c.	Temperature Range and Mean Temperature in ° F.		Results of Post-mortem Examination.		Inoculum.	
			Test.	Control.	Test.	Control.	Test.	Control.
6	3	<i>Per os</i> 5.0	R. 99.4°-103.5° M. 101.7°	R. 100.9°-104.3° M. 102.0°	No lesions	No lesions	5 per cent. desiccated S.F. spleen suspension Ditto	5 per cent. desiccated normal pig spleen suspension Ditto
3	3	Subcutaneous 5.0	R. 100.8°-105.0° M. 102.8°	R. 100.6°-103.5° M. 102.2°	Pasteurellosis in one guinea-pig	Pasteurellosis in one guinea-pig Ditto	Supernatant liquid from above suspension 5 per cent. desiccated S.F. spleen suspension Ditto	Supernatant liquid from above suspension 5 per cent. desiccated normal pig spleen suspension Ditto
3	3	Intradermal 1.0	R. 101.0°-103.3° M. 102.5°	R. 101.4°-103.2° M. 102.2°	Ditto	Ditto	Supernatant liquid from above suspension 5 per cent. desiccated S.F. spleen suspension Ditto	Supernatant liquid from above suspension 5 per cent. desiccated normal pig spleen suspension Ditto
6	3	Intraperitoneal 5.0	R. 100.6°-104.0° M. 102.2°	R. 99.6°-102.8° M. 101.3°	1 Pasteurellosis 1 Pyelonephritis	No lesions	Corresponding brain suspension	Corresponding brain suspension
6	3	Intranasal 0.3	R. 101.2°-104.1° M. 102.6°	R. 100.5°-103.0° M. 101.6°	Pneumonia in one guinea-pig Tuberculous kidney (bovine type) in one guinea-pig	Ditto	Corresponding brain suspension	Corresponding brain suspension
6	3	Ditto 0.3	R. 101.0°-105.7° M. 102.7°	R. 101.3°-104.6° M. 102.5°	1 Pasteurellosis 1 Pyelonephritis 1 Tuberculous spleen (bovine type)	Ditto	5 per cent. desiccated S.F. spleen supernatant liquid	5 per cent. desiccated normal pig spleen supernatant liquid
6	6	Intracerebral 0.1	R. 99.4°-104.1° M. 102.5°	R. 95.0°-104.1° M. 102.6°	No lesions	Ditto	Corresponding brain supernatant liquid	Corresponding brain supernatant liquid
6	6	Ditto 0.1	R. 100.6°-103.6° M. 101.8°	R. 99.4°-103.6° M. 102.1°	No lesions	Ditto	Corresponding brain supernatant liquid	Corresponding brain supernatant liquid

autopsy, bacteriological examination was negative and virus was not recovered from the brain. The other 15 ferrets remained without signs of infection during three weeks' observation and no lesions were found at autopsy after a further four weeks.

White Rats.—Of 42 rats, to which swine fever virus was administered by various routes in the form of the supernatant liquid from desiccated spleen or brain suspension, one developed nervous symptoms on the eighth day after intracerebral injection with the supernatant liquid from swine fever brain suspension and was destroyed on the eleventh day. No virus was recovered from its brain. No symptoms of infection were seen in the remaining rats during the period of observation and all the organs appeared normal at autopsy after six weeks.

Rabbits.—Forty-two rabbits, to which the supernatant liquid from swine fever desiccated spleen or brain suspension was administered by various routes, remained normal during three weeks' observation with no significant difference in mean temperature and temperature range from the control rabbits to which supernatant liquid from the corresponding normal pig tissue suspension had been administered by the same routes. No *post-mortem* lesions were found in any organ after six weeks.

HISTOLOGICAL EXAMINATION

General Procedure.—A normal animal of each species used in the investigation was killed with ether and its brain was removed with aseptic precautions and fixed in 10 per cent. formol-saline. Transverse sections of these normal brains made in the region of the site of inoculation in the test and control animals were first examined microscopically, after staining by haematin and eosin and by Gram's method, to acquire a picture of the normal histology.

Six animals from each species were injected intracerebrally with 0.05 to 0.1 c.c. of the supernatant liquid from a 5 per cent. desiccated swine fever spleen suspension and two with that from 5 per cent. desiccated normal pig spleen suspension. Each inoculum was bacteriologically sterile. One test animal was destroyed in the same manner as the normal at daily intervals up to the fourth day and on the sixth and eighth days, and one control animal one and three days after injection. Their brains were removed, preserved and sectioned as for the normal brains and microscopic examinations were made for significant pathological differences between test and control brain sections.

Results

Mice.—The brain sections of the test mouse, destroyed one day after intracerebral injection, showed marked infiltration of the perivascular connective tissue of the larger cerebral vessels in the vicinity of the third ventricle, with polymorphs, histiocytes and small and large lymphocytes. This feature was absent from all the subsequent

brains in the test mice, although visible to a slight degree in the brain section of the control mouse destroyed one day after injection.

Hamsters.—The brain sections of the test hamster killed one day after injection, differed from those of the corresponding control hamster, only with regard to the degree of hyperaemia of the meningeal and cerebral vessels. Those of the test hamster killed after three days, showed an infiltration of the perivascular connective tissue of the meningeal vessels and of the cerebral vessels near the third ventricle, with small and large lymphocytes and histiocytes, while those of the corresponding control hamster showed no reaction. The reaction seen in the brain sections of the third day test hamster, was still perceptible, although to a less degree, in those of the test hamsters destroyed six and eight days after injection.

Ferrets.—The brain sections of the test ferret destroyed one day after intracerebral injection showed infiltration of the perivascular connective tissue of the meningeal vessels and of the cerebral vessels near the third ventricle with histiocytes and lymphocytes. This reaction was more pronounced in the brain sections of the test ferret killed on the second day. The brain sections of the corresponding control ferrets showed the same type of reaction as seen in the test ferrets but to a much less degree. Little or no reaction was visible in the brain sections of the test ferrets destroyed after six and eight days.

White Rats.—Infiltration of haemoglobin-filled polymorphs and a few eosinophils into the perivascular connective tissue of the meningeal vessels and of the cerebral vessels around the third ventricle was apparent in the brain sections of the test rat killed 24 hours after intracerebral injection. Those of the corresponding control rat showed only some degree of meningeal and cerebral hyperaemia, visible to a less extent in the brain sections of both test and control rats killed at later stages.

Rabbits.—The brain sections of the test rabbits destroyed at daily intervals after intracerebral injection showed varying degrees of infiltration of the meningeal perivascular connective tissue with small and large lymphocytes and histiocytes, most marked in the test rabbit killed on the sixth day. Those of the test rabbit killed on the first day showed infiltration of the same tissue with haemoglobin-filled polymorphs as well. There was a slight leucocytosis of the meningeal veins with haemoglobin-filled polymorphs in the corresponding control rabbit brain.

Guinea-pigs.—The brain sections of the test guinea-pig destroyed on the first day after intracerebral injection showed infiltration of histiocytes, lymphocytes and haemoglobin-filled polymorphs into the perivascular tissue of the meningeal vessels and of the cerebral vessels around the third ventricle. There was a similar but more pronounced reaction in the brain sections of the test guinea-pig destroyed after two days. A leucocytosis of the meningeal and cerebral veins with haemoglobin-filled polymorphs was evident in

the brain sections of the test guinea-pigs killed after three, four, six and eight days.

The brain sections of the control guinea-pigs killed after one and two days showed a similar but much less intense meningeal reaction than those of the corresponding test guinea-pigs. Leucocytosis of the cerebral veins with haemoglobin-filled polymorphs was also seen in the brain sections of the control guinea-pig destroyed after two days and to a less degree in the control guinea-pig, killed after three days.

DISCUSSION OF HISTOLOGICAL RESULTS

Although the meningo-cerebral reaction seen in the brain sections of all the test experimental animals used was usually more pronounced than that seen in the control brain sections, the difference could not be regarded as sufficiently significant to be of practical diagnostic value, and the brains of a much greater number of test and control animals would have to be subjected to microscopic examination to estimate the constancy of the difference in reaction. A method of concentration of purified virus in a small inoculum must be evolved before clear-cut results can be hoped for in view of the probability that the major part of the reaction in the test brains is due to the tissue rather than the virus content of the inoculum. The longer duration of the meningo-cerebral reaction in the test rabbits and guinea-pigs recommends these animals for future experimentation in this field of research.

SUMMARY AND CONCLUSIONS

A study was made in mice, hamsters, ferrets, white rats, guinea-pigs and rabbits, of clinical symptoms following the administration by various routes of swine fever tissue suspension, virulent to a high titre for pigs, and of the histological reaction in the brain after intracerebral inoculation.

Not one of the species of small experimental animals used in this investigation exhibited either clinical symptoms or cerebral reaction of any practical value in the diagnosis of swine fever in the laboratory.

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ON A NEW TYPE OF TOXIN PRODUCED BY *CLOSTRIDIUM WELCHII*

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INTRODUCTION

By comparing the antigenic properties of culture filtrates from several strains of *Cl. welchii*, Wilsdon (1931) was able to identify four toxino-genic types which he designated A, B, C and D. In his conclusions he admitted that "the suggested classification may not be complete in itself since there may exist still further types not represented among the strains used in this investigation." *Cl. welchii* infections in animals are so widely distributed and important that for their diagnosis fresh strains are frequently examined; a very large number therefore have been studied since Wilsdon's article was published but, so far as is known, no additional types have been described with the possible exception of one by Ipsen and his colleagues (1939 *a, b, c, d*) which will be referred to later. The purpose of this communication is to record the isolation of another type and describe the distinguishing properties of its toxin.

Glenny and his colleagues (1933) demonstrated five distinct *welchii* toxins which they designated by letters of the Greek alphabet, α , β , γ , δ and ϵ . The several toxins were differentiated by cross-neutralisation tests with appropriate antitoxins as well as by their capacity to (*a*) haemolyse red blood cells *in vitro*, (*b*) kill mice injected intravenously and (*c*) produce necrosis after injection into the guinea-pig's skin. The relative data are summarised in Table I.

TABLE I
TOXINS OF *Cl. welchii* GROUP

Toxin	Wilsdon's Classification				Haemolysis of Sheep Cells	Lethal Effect in Mice	Dermal Reaction in Guinea-pig
	A	B	C	D			
α	+	+	+	+	+	+	+
β	—	+	+	—	—	+	+
γ	—	+	+	—	—	+	—
δ	—	±	+	—	+	+	—
ϵ	—	+	—	+	—	+	+

+ = toxin produced
— = not produced
± = not invariably produced

+ = reaction. — = no reaction.

Since the above work was published the toxic properties of culture filtrates of so-called *perfringens* strains (*Cl. welchii*, type A) have been further studied. Prigge (1937) reported that in these filtrates he was able to recognise two distinct toxins, namely, a haemolytic factor, α , and a lethal one, ζ . The correctness of his claim that more than one

toxin may be present is now generally recognised but his nomenclature was unfortunate; he overlooked the haemolytic activity of his ζ factor and thus failed to realise its identity with the α toxin of Glenny *et alia* (1933). That this is so seems clear from the work of Ipsen and his colleagues (*loc. cit.*) and also of Macfarlane, Oakley and Anderson (1941), who maintain that ζ is a synonym since the symbol α had already been employed to designate this particular toxin. The letter θ has been used by the latter workers since 1938 to indicate the toxin which Prigge erroneously called α . The position has recently been reviewed by a group of research workers who met to discuss the nomenclature of *Cl. welchii* type A "toxins" and issued a report in *Nature* (1942) defining the characters of α and θ toxins. They evidently concluded that these terms should be used in the sense indicated by Macfarlane, Oakley and Anderson. The main reactions of the toxins were stated to be as follows:—

α Toxin is the main toxic element found in type A filtrates. It is lethal to mice inoculated intravenously, produces a characteristic necrosis on intradermal injection into guinea-pigs and is haemolytic *in vitro* for sheep red cells, the haemolysin being oxygen-stable and requiring the presence of calcium ions for the full development of its action. The toxin reacts specifically with normal human serum and with egg-yolk solution, giving rise to a characteristic turbidity.

θ Toxin exhibits a slight but irregular action in mice after intravenous inoculation because of the natural antitoxin in them, which is also present in other experimental animals. It produces a haemorrhagic reaction on intradermal injection into guinea-pigs but it is not necrotising in action. It is haemolytic towards sheep red cells *in vitro*: the haemolysin is reversibly oxidisable and its activity does not depend upon the presence of calcium ions. It does not display a reaction with human serum or egg-yolk solution.

The workers who drew up this report must have felt that the time was not ripe to pronounce upon a third type of toxin η described by Ipsen and his colleagues (*loc. cit.*) as being lethal but not haemolytic or necrotising in action, but they seem to have left the matter open with the words "*no assumption is made that these two elements*" [*i.e.*, α and θ], "*are the only toxins present in filtrates from all strains of Cl. welchii type A.*" Eventually it may be decided that a strain which produces η toxin should be regarded as belonging not to type A but to one which is additional to those described by Wilsdon.

It may be noted that Oakley and Warrack (1941) state that θ toxin is lethal and necrotising when a sufficiently strong concentration is used for test but they find it cannot be titrated by injection of toxin-antitoxin mixtures intravenously into mice or intradermally into guinea-pigs for mice and guinea-pigs always show a high concentration of circulating antibody; this was found to apply sometimes to other species, in the few animals examined by them. The formation of θ toxin by *Cl. welchii* strains other than type A seems not to have been recorded, but Mr. A. T. Glenny (personal communication) reports that horses immunised with filtrates from types B and C produce some θ antitoxin.

SOURCE AND ISOLATION OF THE ORGANISM

The strain was isolated from the small intestine of a four-days-old calf found dead after an illness lasting about twelve hours during which it showed dullness, inappetence, a slightly subnormal temperature, and signs of general intoxication and collapse. The owner had lost two other calves, both under a week old, in similar circumstances a short time previously.

The *post-mortem* findings in these three animals were as follows :—

Case I.—The mucous membrane of the abomasum was reddened in patches along the edges of the folds ; that of the small intestine was swollen and congested from the duodenum to the middle of the ileum. The contents of this region were brownish yellow and very mucoid. The large bowel appeared normal. There were petechial haemorrhages in the duodenal mucosa and in the mesentery, and larger haemorrhages beneath the epicardium and pleural covering of the lung. The pericardial sac contained a slight excess of fluid.

The heart blood, spleen and mesenteric glands were found to be sterile on culture. The intestinal contents were not examined for the presence of *Cl. welchii* toxin and the isolation of the organism was not attempted.

Case II.—The abomasum was filled with orange-coloured clots of colostrum and its mucous membrane showed numerous petechiae which were especially abundant about 3 inches from the pylorus. The small intestine was intensely reddened throughout its length and filled with pale yellow mucoid material. Several subpleural and interlobular haemorrhages were found in the lungs which were otherwise normal ; aspirated stomach contents were found in the bronchial passages. The liver and kidneys showed cloudy swelling.

Histological examination revealed central necrosis of some liver lobules and larger necrosed areas involving two or three lobules. The intestinal lesions took the form of intense capillary congestion with extravasation of red cells throughout the mucous membrane but there was no evidence of inflammatory reaction.

Smears of the contents of the small intestine showed numerous Gram-positive non-sporulating rods resembling *Cl. welchii* and many Gram-negative coliform organisms. Considering the symptoms and *post-mortem* appearances and the fact that in the first calf no bacterial invasion of the tissues could be detected, the large numbers of the former bacteria found in this situation within a short time of death suggested entero-toxaemia. Mouse inoculation tests showed that a toxic agent was present in the intestinal contents, in such low concentration, however, that it could not be identified. Cultures from the intestine yielded mixed growths among which there appeared to be *Cl. welchii*, but attempts to isolate it were not successful. The centrifuged fluid from such a culture in cooked meat medium proved fatal to mice inoculated intravenously. No protection against it, however, was afforded by any of the *Cl. welchii* antisera ordinarily used for typing* and so the nature of the toxin remained undetermined.

Cultures from the heart blood and bone marrow yielded no growth.

Case III.—Showed well marked enteritis of the small intestine, caecum and colon, petechial haemorrhages in the abomasal mucosa, mesentery, and

* Available through the courtesy of the Wellcome Research Laboratories. For antitoxin content see page 250.

epicardium. The heart blood and bone marrow yielded no growth on culture. The small intestine contained a brown slightly mucoid fluid in smears of which were seen many Gram-positive rods resembling *Cl. welchii*, coliform organisms, and cocci. Centrifuged supernatant fluid of the intestinal contents mixed with an equal volume of saline was lethal to mice injected intravenously in a dose of 0.1 c.c. Its effect was not neutralised by the "standard" *Cl. welchii* sera. An organism indistinguishable from *Cl. welchii* was, however, isolated from the intestinal contents and eight colonies were selected for examination. All of them produced the characteristic type of toxin described below. The strain was labelled No. 6 for laboratory purposes. Two other strains, apparently of the same type, have been isolated recently from the faeces of normal calves.

CHARACTERS OF THE ORGANISM

From its morphology, cultural characters, and staining reactions the organism appeared to be a typical strain of *Cl. welchii*. Surface colonies on blood-agar plates after 18 hours' incubation at 37° C. were surrounded by a zone of haemolysis 1 mm. wide, but only a small proportion showed the smooth, circular, low-convex appearance and entire edge usually associated with young colonies of a freshly isolated strain on this medium. The majority were somewhat irregular in shape with a raised central portion and undulating peripheral zone, the edge of which was irregularly indented and showed here and there a bud-like projection or wavy outgrowth. A somewhat similar form of colony has been noted by McGaughey (1933) and other workers among variants of *Cl. welchii*. The organism produced acid and gas from glucose, lactose, maltose, saccharose, salicin and glycerol but did not ferment inulin, mannitol or dulcitol. It gave rise to typical stormy fermentation in litmus milk followed by partial digestion of the clot, liquefied gelatin, and produced definite though not extensive pitting of the surface when grown on coagulated serum and Dorset egg medium. In cooked meat medium no evidence of digestion was observed and the meat was turned pink in colour.

Mice and guinea-pigs succumbed in 24 to 48 hours to the intramuscular injection of small doses of a young culture in cooked meat medium and showed a massive bloodstained gelatinous oedema at the site of inoculation spreading forward along the belly to the axillary region. The muscles of the affected area had a sodden, partially digested appearance; small bubbles of gas were distributed throughout the lesions which had a slightly butyric, non-putrid smell. Rabbits were more resistant and showed only a local reaction to a somewhat large dose (2 c.c.).

TOXIN PRODUCTION

Mice inoculated intravenously with the filtrate of an 18-hour culture in cooked meat medium died within one to 24 hours, the interval depending on the dose; most of them showed haematuria shortly before death. No protection against the lethal effect was

afforded by mixing the filtrate beforehand with *Cl. welchii* antisera, types A, C or D, or with C and D combined.

This result has been confirmed by Mr. A. T. Glenney who kindly tested for me some five to ten samples each of routine type A, B, C and D sera from horses and found that they gave no protection.

TABLE II
INTRAVENOUS INOCULATION OF MICE WITH MIXTURES OF STRAIN 6 TOXIN
AND *Cl. welchii* SERA

Mixture		Result			
Filtrate	Serum				
0.4 c.c. (8 M.L.D.)	0.1 c.c. Type A	+	+	+	+
" "	" C	+	+	+	+
" "	" D	+	+	+	+
" "	" C and D (equal parts)	+	+	+	+

+ = One mouse died.

Death was rather more delayed on the whole in mice receiving the mixtures than in the controls and was not accompanied by haematuria. Probably, therefore, the filtrate contained at least one toxic factor which was neutralised by the antisera and at least one other against which they were ineffective. The latter component was shown to be antigenic when inoculated into rabbits, six of which were given a course of injections first of filtrate treated as in the preparation of alum-precipitated toxoid and then of the untreated product. A serum was thus obtained which afforded full protection to mice against the action of the filtrate. This serum, therefore, contained an antitoxin which was not present in the "standard" *Cl. welchii* sera; consequently, the corresponding toxin might possibly be a new variety. Steps were therefore taken to determine the properties of this toxin so that it might be compared with the known toxins of the *welchii* group.

METHODS

As Dalling and Ross (1938) have stressed, there are optimal cultural conditions for the production of each *welchii* toxin; unless these conditions are provided a parent strain may fail to yield a measurable quantity of one or other of the toxin types which it is capable of producing. Strain 6 has been grown during periods extending from three hours to ten days in different media and pH values (6.8 to 7.8) to determine its toxin-producing power. Most use has been made of cooked meat medium composed either of beef infusion broth with the addition of peptone and cooked beef mince or Hartley's digest broth plus cooked horse-flesh particles. The effect was tried of varying the proportion of meat particles to broth (20 to 50 per cent. by volume) and of adding glucose (0.1 to 2 per

cent.). Other media tried were infusion or digest broth without meat particles but with glucose and/or serum added and the peptone medium recommended to Crook (1942) by R. S. Roberts for the production of α toxin. The cultures were tested by the haemolytic, mouse-intravenous, and guinea-pig intradermal methods following the technique of Glenny and his colleagues as outlined by Dalling and Ross (1938). The sera employed in testing the filtrates were those of *Cl. welchii* types A, C and D (Wellcome Research Laboratories) and three samples prepared from the homologous strain, of which No. 1 was home-produced from rabbits and Nos. 2 and 3 were experimental yields, from horses, kindly prepared and standardised for the writer by Mr. A. T. Glenny and Dr. R. F. Montgomerie of the Wellcome Research Laboratories. Of the latter No. 2 had a relatively high titre of α antitoxin which showed that the strain had produced a considerable amount of α toxin. No. 3 was obtained by the injection into a horse with no normal α antitoxin of a filtrate to which sufficient α antitoxin had been added to neutralise all detectable α toxin. The antitoxin content of the sera per c.c. was as follows:—

Type A.	Serum 1.	600 α units (international standard)	
		25 θ	"
	Serum 2.	0.2 α	"
		120 θ	"
Type C.	...	6 α	"
		1,000 β	" (provisional standard)
		58 δ	" (" ")
Type D.	...	80 α	"
		1,000 ϵ	" (" ")
<i>Homologous</i>			
No. 1	...	—	" (not standardised)
No. 2	...	190 α	"
		13 θ	"
		240 ι *	" (provisional standard)
No. 3	...	0.2 α	"
		3 θ	"
		16 ι	"

Mixtures of serum and toxin were left standing at room temperature for one hour before use.

Haemolysin Tests.—That α toxin is formed by strain 6 was suspected from the haematuria seen in mice inoculated with filtrates and this was confirmed by testing the filtrate on (a) a suspension of sheep red cells and (b) a solution of lecitho-vitellin prepared as described by Macfarlane, Oakley and Anderson (1941). The former was haemolysed and the latter developed cloudiness followed by flocculation and the separation of a layer of fat. There was a very close correspondence between the end points in the two tests and both reactions were completely inhibited in the presence of α anti-

* Greek letter iota used to designate a special type of toxin produced by strain 6

toxin. As these results held good for all active batches of filtrates that were tested it seems that α toxin was the only haemolytic agent present. It should be noted, however, that most batches contained not only α toxin. Mouse inoculation proved that they also contained the specific lethal factor of strain 6, this factor showing no haemolytic activity. That the strain may also produce θ haemolysin has been kept in mind but attempts to demonstrate it by direct methods have not been successful. In some filtrates slight decrease in haemolytic activity has been observed in the presence of θ antitoxin but no increase has been noted following reduction with sodium hydro-sulphite, which would have revealed any θ toxin present in the oxidised, inactive state (Todd, 1941). However, the question of whether or not the strain is able to form this haemolysin must be looked upon as an open one pending further attempts, by indirect as well as direct methods, to demonstrate it. The production of α toxin by strain 6 reaches its maximum in about four to six hours and then declines rapidly, *i.e.*, the relation between α toxin production and the period of incubation follows a curve much the same as is seen with other types of *Cl. welchii*.

Intravenous Tests in Mice.—That *Cl. welchii* antisera types A, C and D fail to protect mice against strain 6 filtrate has been depicted in Table II. Sera prepared from this strain, besides being effective against it, proved also capable of neutralising type A, but not types B, C or D filtrates. (Table III.)

TABLE III
PROTECTIVE EFFECT OF STRAIN 6 ANTISERUM AGAINST *Cl. welchii* FILTRATES
(Mice I.V.)

Filtrate		Serum	Result			
Type	Dose					
A	8 M.L.D.	0.1 c.c.	—	—	—	—
B	2 "	"	+	+	+	+
C	2 "	"	+	+	+	+
D	2 "	"	+	+	+	+
Strain 6	8 "	"	—	—	—	—

— = 1 mouse lived. + = died.

From the results shown in Tables II and III one may conclude that strain 6 filtrates contain α toxin and another factor which is lethal to mice. Neutralisation tests with the standard sera show that this latter factor is distinct from β and ϵ toxins and is not produced apparently by any of Wilsdon's *welchii* types. This factor is itself a toxin and, it is suggested, may be distinguished conveniently by the letter ι , in accord with Glenny's system of nomenclature.

Intracutaneous Tests.—When the filtrate was inoculated into the skin of a guinea-pig it gave rise to necrosis; from the nature of the reaction this was thought at first to be due possibly to α toxin. It

was found, however, that the necrotising effect was not neutralised by α antitoxin although the reaction was somewhat modified. The kind of reaction produced by a crude filtrate is, therefore, influenced in some degree by the amount of α toxin which the filtrate contains. The α toxin itself elicits a quick response, namely purple discoloration of the skin followed by necrosis which is readable at the 18th hour, and this tends to obscure the more delayed effect of the second necrotising agent present. The reaction to the latter agent in the absence of α takes the form of necrosis which is just discernible by its pallor at the 18th hour, but is best read on the third day when it is much more distinct and often surrounded by a narrow zone of congestion. Table IV shows the response in guinea-pigs to graded doses of a strain 6 filtrate (M.H.D. $1/32$ c.c.), both alone and neutralised with α antitoxin.

TABLE IV
INFLUENCE OF α ANTITOXIN ON INTRADERMAL REACTIONS OF TWO GUINEA-PIGS
TO A SAMPLE OF STRAIN 6 FILTRATE

Dose	Guinea-pig 1		Guinea-pig 2	
	Filtrate alone	Filtrate + 0.5 unit α Antitoxin	Filtrate alone	Filtrate + 0.5 unit α Antitoxin
0.01 c.c.	5 mm.	Speck	8 mm.	5 mm.
0.02 "	10 "	8 mm.	12 "	12 "
0.03 "	13 "	10 "	14 "	12 "
0.04 "	25 "	16 "	28 "	18 "
Control dose of α toxin 2 M.R.D. approx....	18 mm.	0 mm.	22 mm.	0 mm.

The result clearly indicates the presence of a necrotising factor in addition to α toxin. The latter can have played hardly any part in the reactions to the lower doses as they were less than 1 M.H.D., whilst the reactions to the higher doses in the presence of α antitoxin were quite definite. Moreover, filtrates of old cultures produced typical necrosis although they contained no measurable haemolysin. As β and ϵ antitoxins failed to protect mice against the lethal action of filtrate it seemed improbable that either of these toxins was concerned in the skin reactions. As a check, however, mixtures containing 2 and 4 M.R.D. of filtrate with 50 units of β or ϵ antitoxin or both were inoculated into guinea-pigs. The reactions to these mixtures were almost as well marked as to corresponding amounts of filtrate mixed with saline. No reactions occurred, however, on admixture of the filtrate with strain 6 antiserum. Conversely, this serum was without effect on the reactions produced by β and ϵ toxins. The results of the tests upon guinea-pigs were, therefore, consistent with those upon mice in that in both cases the filtrate produced a reaction which was not eliminated by representative *welchii* antisera.

GENERAL CHARACTERS OF ϵ TOXIN

This toxin is manifested by its lethal and necrotising effects. Moderately good yields of it have been obtained in cooked meat medium (pH 7.0 to 7.6). The M.L.D. has varied from 0.025 to 0.1 c.c., the maximum concentration occurring after incubation for 18 to 24 hours at $37^{\circ}C$. The fall in pH during that time is usually only slight. If incubation is continued the toxin level remains practically constant for some time; two batches which were tested at intervals until the eleventh day showed no loss of potency. A batch exposed on the laboratory bench during summer retained its original titre for three weeks, beyond which it was not tested. The toxin has been stored in the cold for nearly twelve months without appreciable loss of potency and in the dried form for two years. On the other hand, it is distinctly heat-labile and is rendered inert on exposure to a temperature of $65^{\circ}C$. for 30 minutes. It is converted into toxoid when mixed with 0.25 per cent. formalin and incubated for two to three days at $37^{\circ}C$. It does not share with ϵ toxin the peculiar feature of becoming greatly enhanced in potency by exposure to trypsin which was reported on by Bosworth and Glover (1934-5). In fact, no increase was observed as the result of such exposure for two hours at room temperature. When inoculated into mice intravenously the toxin causes powerful and rather prolonged, intermittent, muscular spasms affecting particularly the hind limbs, back, and tail. During the intervals the hind legs in some cases become powerless and are dragged along when the animal moves. No haematuria is produced unless α toxin is also present. Death occurs in one to 24 hours, rarely later, depending on the dose.

It has been shown that ϵ toxin and antitoxin can be titrated with reasonable accuracy in mice intravenously. For example:—

<i>Toxin</i>	<i>Antitoxin</i>	<i>Died</i>	<i>Lived</i>	<i>Percentage deaths</i>
0.2 c.c.	1/250 c.c.	5	11	31
"	1/260 "	9	6	60
"	1/280 "	13	2	86.6

A similar degree of accuracy can be obtained by intradermal injection into guinea-pigs.

DISCUSSION

It has been shown that strain 6 filtrate possesses haemolytic, lethal, and necrotising properties. Reasons have been advanced to support the view that the first of these effects is attributable solely to α toxin. The other two effects are caused only in part by this toxin, since they are still produced by filtrates after treatment with α antitoxin and by filtrates obtained from cultures that have been

grown for a long time. There is no need to postulate the existence of more than one factor to explain the two effects since the lethal power of filtrates was found to correspond very closely with their necrotising capacity. Other toxins of the *welchii* group with the same attributes are β and ϵ , but they are ruled out by the results of cross-neutralisation tests. In fact, the work has failed to reveal the presence in strain 6 filtrates of any hitherto recognised *welchii* toxin other than α and it has been concluded that the toxin mainly produced by the strain is a fresh variety. The strain itself may be regarded meanwhile as representing a type which is additional to the types classified by Wilsdon.

Although the circumstances in which the organism was found to occur suggest a causal pathological connection it is by no means proved that it was actually the cause of the calves' illness. It is true that the symptoms and lesions were not inconsistent with a diagnosis of enterotoxaemia and this was supported by the finding of *Cl. welchii* in large numbers in the upper part of the intestine shortly after death. The intestinal contents of two of the three animals were tested and found to be lethal to mice but the nature of the toxic agent was not determined in either case and the evidence is therefore inconclusive. It is possible, of course, that the agent concerned was strain 6 toxin which was afterwards detected in cultures from one of these cases and that this toxin was not identified in the intestinal contents because the corresponding antitoxin was not then available. There is, at present, however, no evidence to show that strain 6 is a cause of enterotoxaemia. Attempts have been made to reproduce the disease in calves a few days old by administering the organism according to the method which was successfully used by Roberts (1938) to set up enterotoxaemia in lambs with *Cl. welchii* type D. These experiments have yielded negative results but they will be repeated to see if still younger calves are susceptible. Since the organism was isolated attention has been paid to the types of *Cl. welchii* present in the intestine of calves sent to the Institute for diagnosis with a history of scouring or other signs of intestinal disorder, but only type A strains have been recovered as yet. The fact that two other strains which produce ι toxin have been isolated from the faeces of normal calves has already been mentioned in the text.

SUMMARY

From one of three calves, dead after showing symptoms of enterotoxaemia, a strain of *Cl. welchii* has been isolated which does not fall into any of the types described by Wilsdon. It produces α toxin in addition to a hitherto unidentified factor, here designated ι toxin. The latter toxin kills mice when injected intravenously and produces necrosis of the skin of guinea-pigs when injected intradermally but does not haemolyse red blood cells *in vitro*. Its possible relationship to disease in calves is discussed.

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THE ESTIMATION OF GLYCEROL IN JOHNIN

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EXAMINATION of a number of batches of johnin prepared either in the complex medium devised by Dunkin (1928) or in his modification of Henley's synthetic medium (1933) and subsequently stored at 0° C. for varying periods, revealed in some a marked decline in potency which did not appear to be obviously related to the age of the product. Although there were fairly wide variations in the pH values of the samples within a range of pH 6.5 to pH 7.5, the loss in activity did not seem to show any connection with the hydrogen-ion concentration. Marked differences were observed, however, in the specific gravity of these samples and some correlation appeared to exist between this factor and activity, in that the johnins of low specific gravity were, in general, also of poor potency. It was thought that the variations in specific gravity might be explained on the grounds that the samples contained different concentrations of glycerol. The present investigation was, therefore, undertaken to determine whether the amount of residual glycerol in samples of johnin could be assayed with any degree of accuracy and, if so, whether the rate of disappearance of glycerol brought about by the growth of *Mycobact. johnnei* on a liquid synthetic medium, could be followed.

Several investigators have studied the utilisation of glycerol by *M. tuberculosis* grown in suitable media. Although satisfactory films are obtainable on glycerol-free veal broth (Allen, 1931), glycerol appears to be necessary when synthetic media are used. Thus Dingle and Weinzirl (1932) state that growth was not inhibited when asparagin was omitted from synthetic medium, but that growth failed when glycerol was omitted. It is generally agreed that glycerol is broken down by acid-fast organisms, the rate of destruction depending to some extent on the presence or absence of other ingredients, such as dextrose. According to Dingle and Weinzirl (1932), there is rapid disappearance of glycerol after the 28th day of incubation and almost all has been utilised by the end of the tenth week. Wedum (1936) also asserts that very little glycerol is left after several weeks' incubation and states that all strains of *M. tuberculosis* utilise as much as 96 per cent. of the oxidisable matter, the glycerol being completely converted into CO₂ and H₂O. Henley and le Duc (1939) agree with Wedum since they found that regardless of the amount of N provided, practically all the glycerol disappeared. Douglas and Hartley (1934) reported similar findings in respect of low glycerol content of tuberculin prepared in synthetic medium.

The part played by glycerol in synthetic media used for the preparation of johnin has not been extensively examined. Eden and Green (1941), in the course of a study of the chemical changes which take place during the growth of various acid-fast organisms on liquid synthetic medium, examined the reactions of two strains

of *M. johnei*. The organisms were propagated on the medium of Watson and Reid (1935) containing 6 per cent. glycerol and 1 per cent. dextrose. Using the dichromate method, in which all oxidisable matter was returned as glycerol, it was observed that the values fell rapidly at first and then more slowly after maximum growth was reached. A study of Table III in their paper shows that with a fast growing strain, glycerol which had an initial level of 6.58 per cent., was reduced to 0.56 per cent. in 68 days, while with a slow growing strain the figures were 6.58 and 0.52 respectively. It may be mentioned that at these laboratories all attempts to secure subcultures in glycerol-free media have been unsuccessful, notwithstanding the use of strains which have been well trained to grow on medium without *M. phlei*. Preliminary experiments have also shown that, in the absence of glycerol, various carbohydrates such as dextrose, maltose, etc., are inadequate. It appears, therefore, that glycerol must be regarded as an essential constituent for cultures of *M. johnei*.

It is probable, however, that like the bovine strains of *M. tuberculosis*, Johne's bacillus will not tolerate high concentrations of glycerol. Thus Eden and Green (1941) have reported that the continuous sub-cultivation of one strain on B.A.I. medium (containing 1 per cent. dextrose and 10 per cent. glycerol) resulted in a progressive decline in the amount of growth. Whether this was due to the presence of the carbohydrate or to the high glycerol content is not clear, but as satisfactory growths were obtained on the Watson-Reid medium which also contains dextrose, it is probable that the glycerol exerted an inhibiting action.

Methods.—In the present investigation the medium of Henley (1929) as slightly modified by Dunkin (1933) was used. Its composition is $K_2HPO_4 \cdot 3H_2O$ 1.0 g., sodium citrate 0.5 g., $MgSO_4 \cdot 7H_2O$ 1.0 g., asparagin 5.0 g., ferric citrate 0.06 g., glycerol 70 ml., and distilled water to 1,000 ml. In most instances the "Teps" (Dunkin, 1933) strain of *M. johnei* has been used since it grows well on this medium, a luxurious surface film being formed within six to eight weeks. The medium was distributed in 250 ml. quantities in one-litre Erlenmeyer flasks which were sterilised by autoclaving.

Determination of Glycerol.—Early methods of estimating the quantity of glycerol in liquid cultures of *M. tuberculosis* were based on specific gravity determinations.

Dingle and Weinzirl (1932) adopted the analytical process of the American Association of Agricultural Chemists in which the fluid under test is evaporated to a small bulk and subsequently extracted with boiling alcohol and boiling ether: the glycerol is determined gravimetrically. It is admitted that this procedure entailed a considerable error due to the fact that glycerol is somewhat volatile at 90° to 100° C., but the results were considered to be sufficiently accurate for an approximate determination. Douglas and Hartley (1934) found that two samples of old tuberculin prepared from glycerol veal broth had specific gravities of 1.135 and 1.145, respectively, which are very close to the specific gravity of a 50 per cent. solution of

glycerol in water, viz., 1:140: on the other hand, two samples of synthetic medium tuberculin showed values of 1.044 and 1.041, respectively, an indication that a high proportion of the glycerol had disappeared during growth. On the basis of specific gravity readings they determined the amount of glycerol which should be added to samples of synthetic medium tuberculin to bring the content to 50 per cent.

Wedum (1936) estimated glycerol by a slight modification of the potassium dichromate method, details of which are described by Allen *et al.* (1940). Henley and le Duc (1939), Eden and Green (1941) also used the dichromate reaction for a determination of the oxidisable organic matter and this was reported as glycerol.

An improvement in the accurate estimation of glycerol has recently been introduced by the periodic acid method elaborated by Malaprade (1928) and subsequently modified by other workers [Voris *et al.* (1940), Allen *et al.* (1940), etc.]. The assay is based on the interaction of periodic acid or of potassium periodate and polyhydric alcohols, in which the KIO_4 is reduced to KIO_3 . The excess of the reagent is determined by titration of the iodine liberated on addition of KI, with standard thiosulphate, at a pH of about 4.0-4.5. The test, which is applicable only to very small amounts of glycerol, is considered to be quite sensitive. According to Voris *et al.*, 1 ml. of 0.00435 N/thiosulphate corresponds to 0.5 mg. of potassium periodate or 0.1 mg. of glycerol.

The reagents required are as follows:—

KIO_4 , 1.25 grm. in 1,000 ml. N/10 H_2SO_4 .

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 15 per cent.

Buffer solution.	$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$.	12 grm. }
	10/N H_2SO_4 .	4.2 ml. }
	Distilled water to	100 ml. }

KI, 5 per cent.

N/1 $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$.	0.5 ml. }
Distilled water	86.5 ml. }

To bring a 7 per cent. solution of glycerol within the sensitive range, the original fluid is first diluted with two parts of distilled water and is then made up in a proportion of two parts of the diluted glycerol solution to 68 parts of water. The degree of preliminary dilution of cultures of *M. johnei* depends on the stage of growth and must be determined by a trial run in which three samples, viz., undiluted, 1:1 and 1:2, respectively, are tested after further dilution in the proportion of 2:68.

To titrate the glycerol, the KIO_4 solution, in 10 ml. quantities, is mixed with 1.5, 1.0 and 0.5 ml., respectively, of the diluted glycerol solution: a blank containing KIO_4 only is included. The mixtures are left for exactly 30 minutes at 20°C ., when oxidation is complete. After the addition of three drops of the MgSO_4 solution, NaOH is introduced until a faint cloudiness appears after which N/10 H_2SO_4 is run in to the point at which the turbidity due to the $\text{Mg}(\text{OH})_2$ just disappears. Ten ml. of buffer solution is now added, followed by 2 ml. of the KI solution. The iodine which is liberated is then titrated against the thiosulphate indicator solution,

The amount of glycerol in the original sample can be determined

by the formula $\frac{Y \times X}{Z} \times 0.175$ where Y is the thiosulphate equivalent

in ml., X is the quantity of diluted glycerol in ml., and Z is the amount of preliminary dilution, viz., 0, 1, 2, etc. The average of the three readings (1.5, 1.0 and 0.5 ml. of dilute glycerol) gives the percentage of glycerol in the liquid. A typical example of the titration of two samples of 7 per cent. glycerol in distilled water and two samples of Henley's medium containing the same proportion of glycerol (as shown in Table I), indicates the degree of accuracy which can be attained.

The results obtained were: glycerol in distilled water—actual 7 per cent., estimated 6.9 per cent. and 7.15 per cent.; glycerol in Henley's medium—actual 7 per cent., estimated 7 per cent. and 7.3 per cent. Samples of Henley's medium restored to their original bulk after incubation at 37° C. have been tested from time to time and have not shown a variation exceeding ± 0.3 ml.

LOSS OF GLYCEROL DURING THE GROWTH OF *M. johni*

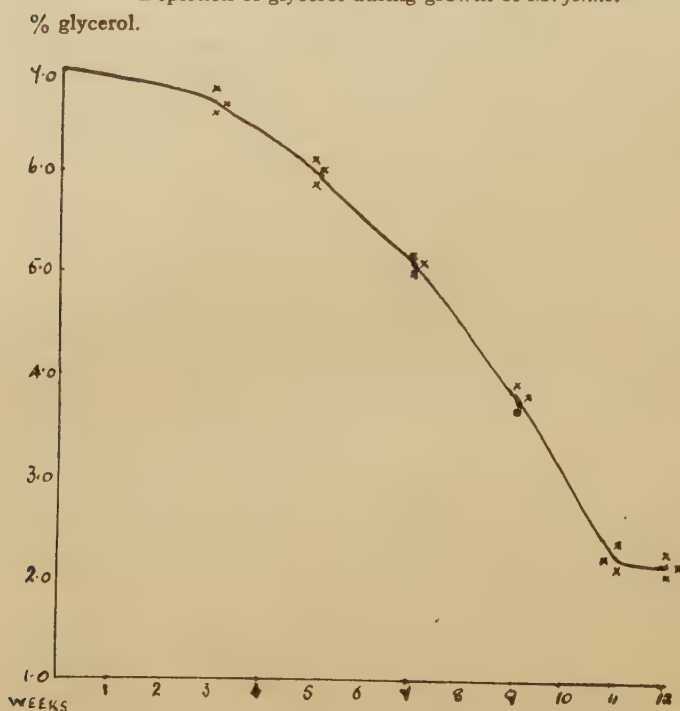
Twelve flasks of Henley's medium were sown with "Teps" strain and incubated at 37° C. Because slight inequalities in the amount of growth are bound to arise in different flasks, a small quantity of fluid (about 20 ml.), was withdrawn from each flask at regular intervals and the concentration of glycerol estimated in the pooled sample. The liquid was steamed for one hour and filtered to remove the bacteria. A preliminary experiment showed that about one-fifth of the fluid evaporated during an incubation period of 12 weeks and that the loss at any given interval was roughly proportional to the time of incubation. Distilled water was therefore added to each sample at the rate of 4.0 ml. per 250 ml. of culture for each week of growth.

Chart I shows the rate of loss of glycerol over a period of 12 weeks. The tests were made after 3, 5, 7, 9, 11 and 12 weeks respectively. Until the third week the glycerol content showed very little fall but by the fifth week it had diminished to 6 per cent. Thenceforward the rate of disappearance was practically constant, the concentration of glycerol falling to 5.1 per cent. at the seventh week, 3.7 per cent. at the ninth week and 2.3 per cent. at the eleventh week. The curve flattens out at this point when the film commenced to sink to the bottom of the flask and growth presumably began to diminish.

A second strain (ARC. 127), which was slightly slower in reaching the peak of its growth, gave similar results, the concentration of residual glycerol after three and a-half months' incubation being 2.08 per cent., i.e., a reduction of a little less than one-third of the original content,

It is interesting that depletion of glycerol proceeds at a somewhat slower rate than seems to occur with *M. tuberculosis*. For example, Dingle and Weinzirl found that one strain of human tubercle bacilli reduced the glycerol content of a synthetic medium from 4.4 per cent. to 0.52 per cent. in nine weeks, while a second strain was even more active since only 1.1 per cent. was detected at the 28th day. Wedum (1936), using the dichromate method, reported that only traces of glycerol were left after various periods of incubation. The results obtained with *M. johnei* are not altogether

CHART 1

Depletion of glycerol during growth of *M. johnei*

x Estimations. 1.5, 1.0, 0.5 ml. dilute glycerol.

unexpected in view of the relative slowness of growth of the organism when compared with the more active mammalian strains of *M. tuberculosis*.

Determination of Glycerol Content of Twelve Samples of Johnin.—To determine the amount of glycerol which remained in johnins when the cultures were harvested, 12 samples were examined. Two (DJ.13, DJ.26) had been prepared in potato-liver-glycerol broth and had been concentrated by heat to one-tenth their volume: a second pair (DJ.38, GJ.19) were samples from synthetic medium,

GJ.19 was not restored to its original volume before it was reduced by heat; no information on this point was available in regard to the other three. In the remaining eight the glycerol content was determined after the johnins had been steamed and sufficient distilled water added to bring them back to their volume before incubation. A small sample from each of the following johnins—J.29, 31, 33, 35, 36 and 42—was then evaporated to one-fifth or one-tenth while the remainder was concentrated in a similar manner after the addition of sufficient glycerol to raise the content to 5 per cent. before heat treatment. JP.34 and JP.37 were used for the preparation of johnin PPD. The results are shown in Table II, from which it

TABLE II
ESTIMATION OF GLYCEROL IN A SERIES OF JOHNNINS

Number of Johnin	Medium	Period of Growth (Weeks)	Per cent. Glycerol after Incuba- tion	Per cent. Glycerol after Concentration		Remarks
				No Glycerol added	Glycerol added to make 5 per cent.	
DJ.13	G.B.	Not known	—	21.6	—	Conc. 1/10
DJ.26	G.B.	Not known	—	16.9	—	Conc. 1/10
DJ.38	S.M.	Not known	—	16.6	—	Conc. 1/10
GJ.19	S.M.	14	—	14.8	—	Conc. 1/10
GJ.29	S.M.	12	2.34	—	52.8	Conc. 1/10
GJ.31	S.M.	13	2.72	12.6	26.6	Conc. 1/5
GJ.33	S.M.	12	1.66	—	24.7	Conc. 1/5
GJ.34	S.M.	12	2.19	—	—	P.P.D.
GJ.35	S.M.	11	2.63	—	24.6	Conc. 1/5
GJ.36	S.M.	13	2.15	11.2	23.8	Conc. 1/5
*GJ.37	S.M.	16	2.08	—	—	P.P.D.
GJ.42	S.M.	13	2.11	19.8	52.1	Conc. 1/10

* Strain other than "teps." G.B. = Potato, liver, glycerol broth.
S.M. = Synthetic medium.

can be seen that the glycerol utilised after 11 to 13 weeks' incubation was represented by a fall of 4.28 to 5.34 per cent. from the original 7 per cent. in the medium.

Accuracy of the Method in the Presence of Other Constituents.—Until now it has been assumed that the reduction of KIO_4 to KIO_3 was solely due to glycerol. There are two factors, however, which

might affect the accuracy of the determinations, viz. (1) certain constituents of the medium itself might react with KIO_4 , and (2) other substances resulting from the metabolic activity of the organism might be formed which might give the same reaction as a polyhydric alcohol and thus lead to a false impression of the amount of residual glycerol. The first hypothesis does not require serious consideration since, as already shown, several batches of Henley's medium gave results which closely paralleled those produced by glycerol alone. It seems safe to assume, therefore, that asparagin, etc., give no appreciable reaction with KIO_4 , thus confirming the lack of interference reported by Wedum when using the dichromate method.

A point arises, however, in connection with the use of dextrose. In the preparation of tuberculin in synthetic medium the formula of Henley modified by Dorset so as to contain 1 per cent. dextrose is widely used. Although so far as is known dextrose is not commonly employed for cultures of *M. johnei*, on general grounds it was thought advisable to check the effect of a carbohydrate on the accuracy of glycerol estimations by the Malaprade method. A series of tests indicated quite clearly that the addition of 10 per cent. dextrose to Henley's medium was responsible for a higher figure for "glycerol" than was obtained in its absence. For example, a sample of Henley's medium alone titrated at 7.06 per cent. glycerol, whereas the same medium with 10 per cent. dextrose gave a figure of 8.20 per cent. : a 10 per cent. solution of dextrose in distilled water was estimated at 2.40 per cent. If, therefore, a large amount of dextrose is added to a medium a correction may have to be made unless the organism utilises the carbohydrate to such an extent that it no longer interferes with the reaction. Actual experiments with media containing decreasing amounts of dextrose proved that with quantities of 5 per cent. or less the difference between the titration figures obtained compared with the figure for glucose-free medium was so slight as to fall almost within the range of the error to be expected when glycerol alone is assayed (Table III).

The second point is concerned with the effect of components which are elaborated during the growth of the organism and pass into solution in the medium. A complete examination of all the constituents formed by *M. johnei* was not attempted but the influence of the protein and carbohydrate fractions on the glycerol reaction has been examined. In a previous paper (Glover, 1941) it was shown that the amount of johnin protein recovered from a series of liquid cultures by precipitation with trichloroacetic acid or by adsorption on benzoic acid varied from 13 mg. to 22 mg. per 100 ml. of original unconcentrated fluid. The carbohydrate complex was obtained from the supernatant fluid, after separation of the protein fraction, by the removal of the excess of acid on a collodion ultrafilter with a constant flow of distilled water followed by the precipitation of the carbohydrate with an equal volume of alcohol. The precipitate was washed with alcohol, dried and weighed. The amounts obtained

varied from 8 mg. to 14 mg. per 100 ml. of original unconcentrated fluid. The effect of johnin protein and johnin carbohydrate on the titration of glycerol was ascertained by preparing batches of Henley's medium with 3 mg. of johnin PPD per ml. and 2 mg. of carbohydrate separately and mixed, representing about 15 times the maximum amount of these constituents found in the cultures after 12 weeks' incubation. As there was no appreciable interference with the glycerol titration (Table III) it is probably safe to assume that the curve shown in Chart I is a true estimate of the glycerol depletion.

Correlation of Glycerol Content with Potency.—As mentioned on page 256, certain batches of johnin which have been kept at cold-room temperature showed an unexpected loss in potency. Some of these have been examined in respect of their glycerol content with the result indicated in Table IV, which also shows the pH value and the specific gravity. In each case the johnin had been stored without any added glycerol to compensate for the amount lost during growth. It is interesting that the two samples of johnin prepared from glycerol veal broth showed a much higher level of glycerol than those obtained from synthetic medium, a result which parallels the observations of Douglas and Hartley on tuberculin. Two johnins (DJ.37 and DJ.44) which at the time of their preparation had shown a satisfactory level of potency, were found, when tested in cattle, to have lost part of their activity since they evoked extremely small reactions in animals which gave a strong response to a standard johnin. The poor activity of these two preparations was confirmed by a guinea-pig test. It will be noted that in both instances the specific gravity was low and an estimate indicated that there was only about 10 per cent. of glycerol remaining in the liquid which had been concentrated by heat to one-tenth of its original volume.

It may be pertinent to enquire whether the addition of glycerol to johnin before heat concentration is likely to have any unfavourable effect on the potency of the finished product. To check this point a flask of synthetic medium sown with "Teps" was steamed at the twelfth week. After restoration of the fluid lost during incubation a determination of the glycerol content gave a value of 2.11 per cent. The liquid was divided into two parts, one of which was immediately concentrated to one-tenth by heat (J.42) while the other (J.43) was reduced in a similar manner after the addition of sufficient glycerol to bring the content to 5 per cent. before evaporation. When tested on the sensitised guinea-pig both samples gave good reactions, J.43 being just appreciably superior to the standard johnin, while J.42 was slightly less active. Both samples are being stored at low temperature and will be tested for potency from time to time.

DISCUSSION

The present study indicates that *M. johnei* grown on Henley's synthetic medium can metabolise about two-thirds of the glycerol.

TABLE IV
COMPARISON OF SPECIFIC GRAVITY, GLYCEROL CONTENT AND BIOLOGICAL ACTIVITY

Date of Preparation	Number of Johnin	Medium	Heat concentration	pH	Specific Gravity	Glycerol determination	Biological Activity	
							Cattle	Guinea-pig
12.6.34	DJ.4	G.V.B.	1/10	7.0	1.090	19.4	++	Not done
19.10.36	DJ.13	G.V.B.	1/10	6.5	1.095	21.6	++	++
16.4.37	DJ.37	S.M.	1/10	6.8	1.055	10.2	±	+
20.5.37	DJ.38	S.M.	1/10	7.6	1.085	16.6	++	Not done
20.8.37	DJ.44	S.M.	1/10	6.6	1.045	9.6	±	+
16.3.38	DJ.48	S.M.	1/10	6.8	1.050	10.5	++	±
26.7.40	GJ.19	S.M.	1/10	6.8	1.080	14.8	Not done	++
20.5.41	GJ.31	S.M.	1/5	6.8	1.075	12.6	++	++
23.2.42	GJ.36	S.M.	1/5	7.2	1.060	11.2	Not done	++
7 per cent. glycerol in Henley's medium.					1.020			
50 per cent. glycerol in Henley's medium.					1.145			

G.V.B. = Glycerol veal broth.
S.M. = Synthetic medium.

Guinea-pig { ++ equal in value to "Standard" johnin.
++ equal 50 per cent. or less than standard.
+ or ± equal less than 20 per cent.

When johnins so derived are concentrated by heat to one-tenth of their original volume the final product contains an average of about 17 per cent. glycerol. These results differ somewhat from those of Eden and Green who found that very little glycerol was left after an incubation period of 68 days. The explanation of this difference may lie in the presence in their medium of glucose which may accelerate the utilisation of glycerol during the period of growth.

Douglas and Hartley (1934) have shown that old tuberculin prepared from synthetic medium may contain very little or no glycerol. In one of their samples there was a very serious and unexpected loss in potency associated with the development of a mould which was subsequently shown to exert a powerful destructive effect on the active fraction. Douglas and Hartley were of the opinion that the well-known stability of O.T. prepared from veal broth did not depend solely on the high concentration of glycerol present since glycerol-free samples suffered no greater loss of activity on heating to 110° C. than similar preparations containing 50 per cent. of glycerol. They strongly urged, however, that sufficient glycerol should be added to synthetic medium tuberculin to act as a bacteriostatic agent and thus indirectly assist in the maintenance of potency.

In the case of the johnins described in this paper, it must be admitted at once that there was no indication of any loss in potency which could be attributed to contamination with bacteria or moulds and no direct evidence was obtained pointing to a low glycerol content being associated with diminished activity. It may be significant, however, that whenever there was a fall in potency in a sample, the johnin in question was also poor in glycerol. On general grounds it would seem advisable to ensure that when synthetic medium johnins are concentrated by heat, the finished products should contain an adequate amount of glycerol. Uniformity can be effected by making a preliminary glycerol determination before heat treatment in the manner described in this paper and by adding the requisite amount of glycerol to bring the final concentration to the desired level which in this investigation has been 5 per cent. before concentration.

CONCLUSIONS

(1) Certain batches of heat-concentrated johnin, grown on liquid synthetic medium, showed loss of potency on storage at low temperature. The loss in activity was not obviously connected with the pH value but was associated with a low specific gravity and a low glycerol content.

(2) The utilisation of glycerol by *M. johnei* during its growth on Henley's synthetic medium was determined by the Malaprade reaction. There was a reduction from an initial level of 7 per cent. to an average of 2.23 per cent. after 11 to 16 weeks' incubation.

(3) The accuracy of the estimation of glycerol by the Malaprade reaction was not affected by dextrose in quantities not exceeding

5 per cent. or by the presence of johnin protein or johnin carbohydrate.

(4) The association of low glycerol content with the loss in potency is discussed and the addition of glycerol to heat concentrated johnins is suggested.

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